Oxidative and nitrative stress biomarkers in amniotic fluid and their association with fetal growth and pregnancy outcomes

Dima El-Halabi
School of Dietetics and Human Nutrition
Macdonald campus
McGill University, Montreal, Quebec

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ABSTRACT

The study objectives were to: 1) assess fetal exposure to oxidative stress by measuring amniotic fluid concentrations of nitric oxide (NO), thiobarbituric acid–reactive substances (TBARS), and ferric reducing antioxidant power (FRAP) and 2) establish whether these concentrations were associated with infant birth weight, gestational age, or oxidative stress-related conditions arising during pregnancy. Frozen amniotic fluid samples were obtained from 654 mothers undergoing amniocentesis for genetic testing during second trimester in Montreal, QC, Canada. Maternal and neonatal characteristics were collected from medical charts and questionnaires and exclusion criteria were applied. ANOVAs and multivariate regression analyses showed that NO, which differed among pre-term, term, and post-term groups, was a positive predictor of gestational age. TBARS were highly correlated with sample storage and were not associated with pregnancy outcome parameters. FRAP positively predicted gender-corrected birth-weight-for-gestational-age. Our study shows that markers of oxidative and nitrative stress in-utero are associated with pregnancy outcomes.

SOMMAIRE

Les objectifs d'étude étaient: 1) évaluer l'exposition foetale au stress oxydatif en mesurant les concentrations en oxyde nitrique (NO), l'acide de thiobarbituric et les substances acido-réactives (TBARS) du liquide amniotique et le pouvoir antioxydant de réduction de l'ion ferrique (FRAP) et 2) établir si ces concentrations sont associées avec le poids de l'enfant à la naissance, l'âge gestationnel, ou à des conditions reliées au stress oxydatif se présentant pendant la grossesse. Les échantillons de liquide amniotique congelés ont été obtenus de 654 mères subissant l'amniocentèse pour le test de dépistage génétique, pendant le deuxième trimestre à Montréal, QC, Canada. Les caractéristiques maternelles et néo-natales ont été recueillies à partir de graphiques et questionnaires médicaux en appliquant les critères d'exclusion. Les analyses de régression ANOVAs et multivariés ont montré que NO, qui a différé entre les groupes pré-terme, à terme et post-terme, était un test de prédiction positif de l'âge gestationnel. Les TBARS ont été significativement associées au stockage de l'échantillon mais pas aux paramètres mesurant les effets de la grossesse. Le FRAP a positivement prédit le poids de naissance pour l'âge gestationnel adapté selon le sexe. Notre étude montre que les marqueurs du stress oxydatif intra-utérins sont associés avec les effets de la grossesse.

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CONTRIBUTION OF AUTHORS

In conjunction with Dr. Kristine G. Koski, the author developed the present research project. The analysis of the amniotic fluid samples for NO, TBARS and FRAP were conducted by the author. Kebba Sebally provided the training for the NO assay and performed a significant proportion of the samples analyzed for FRAP. Dr. Kubow recommended the measurement of lipid peroxidation by the TBARS method and provided assistance with lab procedures for the assay. The author reviewed medical charts, performed the data entry, statistical analysis, and writing of the thesis and manuscript.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

AGA Appropriate-for-gestational-age

ANOVA Analysis of variance

ANCOVA Analysis of covariance

BMI Body mass index

BW Birth weight

eNOS Endothelial nitric oxide synthase

F2-IP F2-Isoprostane

FGR Fetal growth restriction

FRAP Ferric reducing antioxidant power

GDM Gestational diabetes

GA Gestational age

GSH Reduced glutathione

GSH-P_x Glutathione peroxidase

HPLC High performance liquid chromatography

iNOS Inducible nitric oxide synthase

IUGR Intrauterine growth restriction

LBW Low birth weight

LGA Large-for-gestational-age

MDA Malondialdehyde

NO Nitric oxide

NOS Nitric oxide synthase

PIH Pregnancy-induced hypertension

ROS Reactive oxygen species

SOD Superoxide dismutase

SGA Small-for-gestational-age

TBA 2-thiobarbituric acid

TBARS Thiobarbituric acid-reactive substances

VEGF Vascular endothelial growth factor

VLBW Very low birth weight

Wt Weight

CHAPTER 1: OVERVIEW

Oxidative stress occurs when the balance between reactive oxygen species (ROS) and the antioxidant defenses which have the ability to scavenge them is disrupted (Halliwell, 1994). Pregnancy represents a state of oxidative stress due to the increased placental mitochondrial activity and production of ROS (Agarwal *et al.*, 2005). There is some evidence that the levels of lipid peroxides increase in normal pregnancy and that the increase is offset by an increased level of antioxidant vitamins (Wang *et al.*, 1991a). Excessive production of ROS may occur during placental development and pathological pregnancies such as those complicated by preeclampsia, diabetes and IUGR (Myatt *et al.*, 2004), overriding the antioxidants present.

Evidence of oxidative stress may be investigated through the measurement of several different biomarkers, including increased lipid peroxidation products (Wang *et al.*, 1992, Gratacos *et al.*, 1998, Pasaoglu *et al.*, 2004) and decreased expression and activity of antioxidants (Wang *et al.*, 1991b, Davidge *et al.*, 1992, Hubel, 1999, Myatt *et al.*, 2004). Decreased activities of the antioxidants superoxide dismutase (SOD), glutathione peroxidase (GSH-P_x), and vitamin E have been shown to be decreased in placental tissues from preeclamptic pregnancies (Wang and Walsh, 1996). Intrauterine growth restriction (IUGR) is associated with elevated perinatal mortality and morbidity and may be correlated with changes in oxidative stress parameters (Karowicz-Bilinska, 2002, Takagi, 2004). Recent studies also implicate IUGR with adult onset of hypertension, coronary heart disease, hypercholesteremia, and diabetes (Godfrey and Barker, 2000). The evidence for oxidative stress in small-for-gestational-age (SGA) and low birth weight (LBW) infants may lead to effective therapies if predicted earlier in gestation (Gupta *et al.*, 2004).

During normal pregnancy, uterine and systemic vascular functions change dramatically and the maintenance of low vascular tone becomes necessary for fetal health and growth (Rosselli *et al.*, 1998). The human fetal-placental vasculature lacks autonomic innervation, relying on autocrine/paracrine agents such as the nitric oxide (NO) radical to play an important role in the regulation of fetal-placental blood flow (Kossenjans *et al.*, 2000). Various animal studies have shown that the inhibition of nitric oxide synthase (NOS) during pregnancy causes decreased placental and fetal perfusion

and subsequently results in fetal growth restriction (FGR), an implication that NO is closely associated with fetal growth (Yallampalli and Garfield, 1993, Diket *et al.*, 1994, Salas *et al.*, 1995, Helmbrecht *et al.*, 1996).

However, there seem to be many contradictions concerning the involvement of NO in the maternal adaptations to pregnancy. On the one hand, an impairment in NO biosynthesis has been proposed as a possible mechanism for reducing fetoplacental circulation in pregnancies complicated with higher vascular resistance such as preeclampsia and IUGR (Sladek *et al.*, 1997). Hence, an overexpression of endothelial NOS (eNOS) has been widely regarded as an adaptive response to compensate for decreased placental blood flow (Hefler *et al.*, 2001) in such pathological conditions. On the other hand, NO as a free radical can affect vascular function in the face of oxidative/nitrative stress. The expression of nitrotyrosine residues, a marker of NO-superoxide interactions that produce the prooxidant peroxynitrite, have been found in the fetal vasculature of preeclamptic and diabetic pregnancies (Myatt *et al.*, 1996, Norris *et al.*, 1999). Hence, NO can play a dual role with respect to its influence on the feto-placental vasodilation as well as nitrative stress-induced pathological conditions of pregnancy (Morris *et al.*, 1996, Weiner and Thompson, 1997, Lyall *et al.*, 1998).

Amniotic fluid is a unique and complex biological medium that facilitates fetal growth, providing protection and various nutrients and growth factors (Underwood *et al.*, 2005). Based on the significant contributions of amniotic fluid to fetal health, the assessment of its content may provide a window for assessing fetal growth and disease, yet more research is needed in understanding amniotic fluid biology and clinical significance. As there is no single reliable indicator of oxidative stress, a combination of methods may provide the best approach to measure oxidative stress. We chose to investigate three biomarkers of oxidative/nitrative stress in amniotic fluid: NO, thiobarbituric acid –reactive substances (TBARS), and ferric reducing antioxidant power (FRAP) and determine whether they were established with pregnancy outcomes, including infant birth weight and gestational age (GA) as well as oxidative stress induced conditions of pregnancy (gestational diabetes; GDM, pregnancy-induced hypertension; PIH, smoking, obesity).

This study aims to establish indices for monitoring the intrauterine condition of the fetus and lay the foundation for new treatments of adverse pregnancy outcomes. More specifically, the investigation of three different indicators that contribute to the oxidative stress/antioxidant capacity of the amniotic fluid in early gestation may help understand the role they play in influencing and determining fetal and pregnancy outcomes.

CHAPTER 2: LITERATURE REVIEW

2.1. General background on fetal growth

2.1.1. Fetal Growth

Fetal growth is regulated by genetic, placental and maternal factors (Peleg *et al.,* 1998). The maternal-placental-fetal units act in unison to provide the needs of the fetus while supporting the physiological adaptations for the mother. Limitation of growth potential of the fetus is parallel to failure to thrive in the infant. Fetal growth restriction (FGR) is the second leading cause of perinatal morbidity and mortality and has been associated with poor pregnancy outcomes, including fetal and neonatal death, reduced intelligence, seizures, and cerebral palsy (Goldenberg *et al.,* 1990).

Intrauterine growth restriction (IUGR) is defined as a fetus whose estimated weight is below the 10^{th} percentile for its gestational age and whose abdominal circumference is below the 2.5^{th} percentile. Decreased intrauterine growth may have a negative effect on brain growth and mental development potential (Markestad *et al.,* 1997). Low birth weight (LBW) is defined as an infant weighing less than 2500 grams (5.5 lb) and is associated with neonatal morbidity and mortality, developmental problems and other illnesses (Kramer, 1987a,b, National Academy of Sciences, 1990). Research has shown that LBW is a result of multiple factors, including interactions between the biological determinants of the mother and the fetus, the parent's social environment, and the efficacy of medical care during the periconceptual, prenatal, and perinatal periods (Thompson *et al.,* 2005).

A macrosomic baby is defined as weighing more than 4000 grams (8.8 lbs). Fetal macrosomia results from fetal hyperinsulinemia due to increased maternal–fetal transfer of glucose and other nutrients (Schwartz, 1990). Fetal macrosomia could cause complications during delivery such as shoulder dystocia, birth trauma and need of early or preterm delivery or operative delivery (Schwartz, 1990, Weeks *et al.,* 1994, Okun *et al.,* 1997, Wen *et al.,* 2000).

The lowest risk of infant mortality is associated with birth weights between 3500 g- 4000 g (7.7-8.8 lb) (Chase, 1967, Chase, 1969, Saugstad, 1981). Accurate dating in early pregnancy becomes important for diagnosing IUGR. The usual qualifier for the establishment of an accurate gestational age is a certain date for the last menstrual period

in a women with regular cycles or an ultrasound examination performed no later than the 20th gestational week. Among the most predictive factors for IUGR is a previous small-for-gestational age infant (Tejani, 1982, Wolfe *et al.,* 1987).

Preterm birth is defined as birth with a gestational age of less than 37 completed weeks and is the single most important cause of perinatal mortality and morbidity in industrialized countries (Mesleh *et al.,* 2001, Goldenberg, 2002). The known risk factors for preterm birth are: black race, single marital status, younger or older maternal age, previous preterm delivery, smoking, low pre-pregnancy weight, low or high weight gain (Wen *et al.,* 1990) and multiple pregnancy (Joseph *et al.,* 1998, 2002). Post-term birth is defined as birth with a gestational age at birth of 42 or more completed weeks.

A new birth weight classification based on a population reference has been favored over the gram weight classification (Kramer *et al.,* 2001). The new birth weight percentiles correct for gender and gestational age where infants < 10% are classified as small-for-gestational-age (SGA), those between 10 and 90% as appropriate-for-gestational-age (AGA) and those infants ≥90% as large-for-gestational-age (LGA). SGA represents a statistical grouping of infants whose birth weight and/or length is below the 10th percentile for gestational age. The term SGA refers not to fetal growth but to the size of the infant at birth. Among the known risk factors for LGA infants include maternal obesity, maternal diabetes, multiparity, previous gestational diabetes, and previous delivery of an infant weighing 4000 g or more (Modanlou *et al.,* 1980).

Current research suggests that some of the major diseases in adulthood, including coronary heart disease, hypertension, and type 2 diabetes, originate in impaired intrauterine growth and development (Barker *et al.,* 1993a, Godfrey and Barker, 2000). The theory of "fetal origins" or "programming" in which a nutritional insult in early life has permanent effects on structure, physiology and metabolism, is still unresolved. Increased rates of coronary heart disease, hypertension, and diabetes were shown through longitudinal studies of 25,000 UK men and women in which size at birth was associated with the presence of disease in middle age (Barker *et al.,* 1993b). This relationship was independent of the length of gestation; hence attributed more to FGR than premature birth. The link between fetal growth and onset of adult diseases carries strong implications for

the importance of appropriate maternal-feto-placental nutrient supply during pregnancy (Waterland *et al.,* 1999).

2.1.2. Determinants of infant birth weight

Pre-pregnancy weight

Low prepregnancy weight is a strong determinant of both IUGR and prematurity, both of which may result in a LBW infant (Kramer, 1987a). The risk of low pre-pregnancy weight is greatly reduced by an appropriate gestational weight gain between 12.5 and 18.0 kg (28-40 lb) in total, amounting to 0.5 kg (1 lb) per week in the second and third trimester. Based on prepregnancy body mass index (BMI) alone, healthy weight women at a BMI of 20 to 27 are at lowest risk for giving birth to either a LBW or high birth weight infant (National Academy of Sciences, 1990).

Women with a high prepregnancy weight (BMI > 27) are more likely to develop gestational diabetes mellitus (GDM) and to give birth to a macrosomic infant, especially if gestational weight gain is high (Dawes *et al.,* 1991, Johnson *et al.,* 1992). A positive relationship has been shown between maternal prepregnancy weight and neonatal weight-height index in both diabetic women and controls (Vohr *et al.,* 1980). Increases in pre-pregnancy weight and weight gain have been documented in Canada (Canadian Perinatal Health Report, 2003).

Gestational weight gain

There is evidence that women who do not gain enough weight during pregnancy may deliver a LBW infant (Abrams and Laros, 1986). On the other hand, excessive gestational weight gain has been associated with a high birth weight. The presence of a high birth weight, especially greater than 4500 g may lead to prolonged labor and birth, birth trauma, birth asphyxia, caesarean birth and increased risk of perinatal mortality (National Academy of Sciences, 1990). Current recommendations suggest weight gain ranges based on the different categories of pre-pregnancy BMI, which reflect observations of healthy pregnancy outcomes (Canadian Perinatal Health Report, 2003).

Maternal age

Most studies show that numerous complications of pregnancy affecting both the mother and fetus or neonate are associated with advanced maternal age (Kessler *et al.,* 1980, Forman *et al.,* 1984, Strobino *et al.,* 1995). The age of 35 is frequently used as a cutoff, although there is no precise age beyond which a woman becomes more susceptible to those complications, especially in reference to women having their first child (Cunningham and Leveno, 1995). Maternal age has a U-shaped relationship with infant mortality among singletons (Misra and Ananth, 2002); this relationship has become a growing concern due to the growing proportion of first births to older women.

No significant increase in perinatal morbidity was observed by Kessler *et al.* (1980), but the incidences of prematurity and perinatal mortality were higher. There were also no differences seen in the incidences of diabetes, heart disease, and essential hypertension. In contrast, another study (Joseph *et al.,* 2005) showed that older women were more likely to have hypertension, GDM, placental abruption, or placenta previa. Older maternal age was associated with relatively higher risks of perinatal mortality/morbidity, although the absolute rate of such outcomes was low; preterm birth and SGA rates were also higher.

Berkowitz *et al.* (1990) found a slight elevation in the risk of having a LBW infant among primiparous women who were 35 years of age or older as compared to women between 20 and 29 years of age. However, there was no evidence that women between 30 and 34 or older than 35 years of age had an increased risk of delivering a preterm or SGA neonate, having an infant with a low APGAR score or increased risk of perinatal mortality. Delayed childbearing played an important role in low-birth weight trends in the United States (Yang *et al.,* 2000). In a study of 36,056 women (Cleary-Goldman *et al.,* 2005), increasing age was associated with miscarriage, chromosomal abnormalities, congenital anomalies, GDM, placenta previa, and cesarean delivery, while between 35 and 39 years were at increased risk for macrosomia. Increased risk for abruption, preterm delivery, LBW, and perinatal mortality was prominent in women aged 40 years and older.

Among 539 deliveries from older women (aged 50 years and above), the risks for LBW, preterm, and very preterm were three times greater among older mothers, while the incidence of very low birth weight (VLBW), SGA, and fetal mortality occurred two times

more often compared with young mothers. Among multiple gestations, the differences in risk between older and young mothers were lower than those noted among singletons yet compared with young mothers, older mothers had significantly higher risks of LBW, VLBW, very preterm, and SGA (Salihu *et al.,* 2003). Advanced maternal age after 25 years was associated with an increased risk of fetuses with congenital malformations when excluding aneuploidy (Hollier *et al.,* 2000).

Maternal height

Maternal height is a significant determinant of birth weight and growth in childhood (Kramer, 1987a). Maternal height is positively associated with birth weight and birth length (Pickering, 1987, Meis *et al.,* 1997) after 35 weeks of gestation, regardless of maternal race (Witter and Luke, 1991). In a study of 395 newborns, maternal height was found to be positively correlated with birth weight (Mohanty *et al.,* 2006). The increase in the mean maternal height was found to have a significant effect on the observed trend of increasing mean birth singleton birth weights in the United Kingdom (Bonellie and Raab, 1997).

Ethnicity

Ethnic differences in birth weight and fetal growth have been documented (Kramer, 1987a, Frisbie *et al.,* 1997, Dubay *et al.,* 2001). Kramer (1987a) observed that North American whites and Europeans have higher birth weights than Blacks, Pakistanis and Indians, while North African Jews and North American Indians tend to deliver larger babies. Ethnic differences in prenatal characteristics and birth weight outcomes were found between Asian and white women as well as between Asian subgroups in California (Fuentes-Afflick and Hessol, 1997). A study which compared pregnancy outcomes in three ethnic groups: African-American Blacks, non-Hispanic Whites, and Filipinos in Hawaii found higher risks of LBW for Filipinos and Blacks compared with Whites but no ethnic differences in neonatal mortality were found (Alexander *et al.,* 1993). A large body of evidence has demonstrated that infants born to black women in the U.S. are at a higher risk for LBW and preterm delivery than infants born to white women (Friedman *et al.,* 1993, Hessol *et al.,* 1998, Fang *et al.,* 1999, Palotto *et al.,* 2000, Centers for Disease

Control and Prevention, 2002). However, the heterogeneity within racial groups has also lead researchers to believe that cultural, social, and environmental factors coupled with the nativity and ethnicity may also take part as risk factors (James, 1993).

Shiono and Klebanoff (1986) observed a shorter gestational length in certain ethnic groups, in which Blacks had the highest rate of preterm and very preterm delivery, followed by Mexican-Americans, Asians, and Whites. A shorter gestational length of an estimated 5 days was reported in the Black population, suggesting an earlier maturity of the fetoplacental unit (Papiernik *et al.,* 1990, Omigbodun and Adewuyi, 1997). Differences in mean birth weight and gestational length were more strongly related to the mother's rather than the father's race (Migone *et al.,* 1991).

Parity

A review of 4179 pregnancies showed that parity was a significant determinant of birth weight (Gardosi *et al.,* 1992). Ogun *et al.* (1985) found that the average weight at birth tends to be low for low and high parities and that the highest mean weights reported were between parities 3 and 6. Nulliparity and grand multiparity have been associated with an increased risk of delivering a neonate with evidence of FGR (Vorherr, 1982). The incidence of delivering a growth restricted infant is increased if the woman has previously delivered a child with this condition since conditions accounting for FGR in the first pregnancy are likely to be present in subsequent pregnancies (Bernstein *et al.,* 1997, Langer, 2000). A pregnant woman with a history of LBW infants has a 2.75 greater risk of delivering an infant with IUGR (Kramer, 1987a).

Smoking

Cigarette smoking during pregnancy can have adverse health effects on the fetus and infant, increasing the occurrence of IUGR, preterm birth, spontaneous abortion, placental complications, stillbirth, and sudden infant death syndrome (Canadian Perinatal Health Report, 2003). Smoking during pregnancy reduces birth weight by an average of 200 g and may increase risk of preterm delivery and perinatal mortality (Wisborg *et al.,* 1996, Groff *et al.,* 1997). The association between maternal smoking and adverse pregnancy outcomes is related to the amount and duration of smoking. The rate of preterm birth doubles in women who smoke up to 10 cigarettes per day compared to the

rate in non-smokers (Anderson *et al.,* 1984). Smoking during pregnancy had a decreasing effect on birth weight even when correcting for gestational age, gender, and parity in many studies (Kariniemi and Rosti, 1988, Brooke *et al.,* 1989).

Increasing energy intake alone will not prevent fetal growth retardation (Muscati *et al.,* 1996). Women who stop smoking before they become pregnant or during pregnancy have a significantly lower risk of IUGR and preterm birth compared to women who smoke throughout their pregnancy. The negative effect on fetal growth is the greatest during the third trimester (Lumley, 2002, Canadian Perinatal Health Report, 2003). Smoking is strongly associated with low socio-economic state in developed communities and remains one of the strongest associated factors that is easily modifiable (Robinson *et al.,* 2000).

Gestational diabetes

The primary pregnancy complication that is associated with GDM is excessive fetal size or macrosomia (Jarrett, 1993, Okun *et al.,* 1997). Reports that women with GDM have a higher incidence of obstetric complications including pregnancy-induced hypertension, premature membrane rupture, cesarean section and preterm delivery, as well as higher birth weight infants, macrosomia, and LGA have been documented (Magee *et al.,* 1993, Weeks *et al.,* 1994, Solomon *et al.,* 1997, Xiong *et al.,* 2001). There is an increased incidence of LGA, not only in mothers who equal or exceed the threshold values defining GDM on an oral glucose tolerance test (OGTT), but also among demonstrating lower degrees of glucose intolerance (Weiner, 1988). Leikin *et al.* (1987) showed that pregnant women who had high 1-h glucose screening test values and normal OGTT results gave birth to macrosomic babies more often than those with normal glucose screening tests. Green *et al.* (1991) demonstrated a positive relationship between 1-h glucose screening test values and birth weight.

Hedderson *et al.* (2003) observed that the risk of spontaneous preterm birth increased with increasing levels of pregnancy glycemia; this association was independent of perinatal complications, such as preeclampsia–eclampsia and birth weight for gestational age that could have triggered early delivery. Other studies (Tallarigo *et al.,* 1986, Magee *et al.,* 1993) found no association between glucose tolerance and preterm

birth. Women with IGT were at increased risk for premature rupture of membranes (PROM), preterm birth, breech presentation, and high birth weight (90th percentile or 4,000 g) (Yang *et al.,* 2002).

Pregnancy-induced hypertension

The incidence of FGR is increased 2-3 fold in women with chronic hypertension, with severity of the disorder being directly correlated with increasing incidence of IUGR (Scott *et al.,* 1981, Martikainen *et al.,* 1989). However, it is not clear whether this is the result of the angiospastic features of the disorder which cause a reduction in uterine blood flow or the result of the higher presence of preeclampsia in women who have chronic hypertension (Bernstein *et al.,* 1997). In contrast, Redman *et al.* (1976) reported no change in the incidence of FGR among hypertensive women with the control of blood pressure during early pregnancy. A retrospective cohort study on 97,270 pregnancies in Canada investigated the impact of preeclampsia and gestational hypertension on birth weight according to gestational age (Xiong *et al.,* 2001). Lower birth weights were found in mothers with preeclampsia who delivered infants at 37 weeks or less; however they were not lower in the preeclamptic group which delivered after 37 weeks, suggesting that most babies born to preeclamptic mothers at term have normal fetal growth.

Infant gender

Many reports reveal that males are generally heavier than females at each gestational age (Brenner *et al.,* 1976, Alexander *et al.,* 1999, Oken *et al.,* 2003) and are at a higher risk of being born IUGR (Kramer, 1987a). Ogun *et al.* (1985) studied a population of 28,000 live births and found males to be heavier than females at birth. Males also have higher rates of fetal and neonatal mortality than females and are more susceptible to long-term neurological and motor impairments after preterm birth (Smith, 2000, Stevenson *et al.,* 2000). There is also a higher proportion of preterm births among males vs. females, which may explain the higher mortality incidence in infancy (McGregor *et al.*, 1992, Cooperstock and Campbell, 1996, Astolfi and Zonta, 1999).

Gestational age

Both fetal growth and length of gestation contribute to birth weight. A better understanding of the determinants of fetal growth can occur with the removal of the contribution of gestational age (Oken *et al.,* 2003). Birth weight and gestational age have a strong association in normal pregnancies (Langer, 2000). Gestational age accounts for a large variability in birth weight; as the infant matures, it grows (Wilcox and Skjoerven, 1992). Preterm birth is a great predictor of neonatal complications, mortality, and long-term health sequelae (Canadian Perinatal Health Report, 2003).

2.1.3. Role of amniotic fluid in fetal growth

Amniotic fluid, the serous fluid within the amnion that surrounds the fetus, plays a very important role in fetal growth and development. Amniotic fluid serves numerous functions in the growing fetus. These include acting as a shock absorber, allowing for freedom of fetal movement and symmetrical musculoskeletal development, maintaining a relatively constant temperature for the environment surrounding the fetus, reducing infections due to its antibacterial properties, and finally permitting proper lung development (Brace, 1997, Sohaey, 1998). In addition, the fluid provides nutrients and growth factors to the fetus, acting as a source of human fetal nutrition (Mulvihill *et al.,* 1985, Underwood *et al.,* 2005). The nutrients swallowed, digested and absorbed by the fetus, including sugars, proteins, amino acids, and lactate, provide it with an estimated 10-30 calories/day and 0.2-0.3 g protein/kg/day in the third trimester (Gitlin *et al.,* 1972, Lilley, 1972). Based on the variety of functions that amniotic fluid has shown to play in fetal health, further research is needed to appreciate its importance.

The volume and composition of amniotic fluid varies with gestational age (Brace, 1997). During the first half of pregnancy, volume of amniotic fluid increases gradually. In the second half of pregnancy, the level of fluid generally stays the same until the fetus reaches full term around week 39, at which time there is a sharp decline in volume of fluid. On average, the amniotic fluid volume measures 800 ml between 22 and 39 weeks (Sohaey, 1998). Amniotic fluid disturbances characterized by a deficiency in amniotic fluid (oligohydramnios) or an excess of fluid (polyhydramnios) have been associated with

perinatal morbidity and mortality; hence, techniques for evaluation of such abnormalities should be emphasized (Brace, 1997, Sohaey, 1998).

There are multiple pathways for the exchange of fluids in the amniotic cavity, including two primary sources and two primary routes for removal during the second half of gestation (Ross and Nijland, 1997). Fetal swallowing is a major contributor of amniotic fluid regulation with an estimated 200 to 500 ml of fluid being swallowed by the fetus daily towards term (Sohaey, 1998). The other important non-fetal pathway for fluid removal is intramembranous absorption, in which fluid perfuses the placenta directly into the fetal blood (Seeds, 1980, Gilbert and Brace, 1993, Brace, 1997). In addition, transmembranous absorption occurs when fluid crosses the amniotic membranes and enters the maternal vessels lining the uterine wall (Brace, 1997). An understanding of the physiological regulation of amniotic fluid volume (AFV) is important for the ultrasound practitioner in recognizing aberrations in fluid volume.

Alternatively, there are multiple possible sites of formation of amniotic fluid that arise from several physiological processes (Gilbert and Brace, 1993). Immunological evidence suggests that both the fetus and the mother make individual contributions to the amniotic fluid (McCarthy and Saunders, 1978, Modena and Fieni, 2004). In the first trimester, amniotic fluid is a transudate from chorionic trophoblast and fetal tissue (McCarthy and Saunders, 1978). Later, ultra-filtration through the fetal skin is a major source before the skin cornifies at around 23-25 weeks gestation (McCarthy and Saunders, 1978). Then, at the end of the first trimester, amniotic fluid production shifts from transudation to renal secretion, at which time the fetal kidneys begin excreting urine. Hence, the fetal kidneys are the main contributors of amniotic fluid production during the second and third trimesters of pregnancy after which the biochemical components of the fluid become increasingly similar to diluted fetal urine (Lind and Cheyne, 1969). The fetal lungs are another important contributor to amniotic fluid (Brace and Wolf, 1989, Sohaey, 1998). The net amniotic fluid volume results from the balance between fetal fluid production, via lung liquid and urine, and fluid resorption (Ross and Nijland, 1997, Modena and Fieni, 2004).

2.2. Oxidative and nitrative stress, pregnancy and fetal growth

2.2.1. Reactive oxygen species (ROS)

2.2.1.1. ROS and oxidative stress

Free radicals and reactive oxygen species (ROS) are constantly being generated in vivo, both by "accidents of chemistry" and as an integral part of human metabolism (Halliwell, 1994). Free radicals are highly reactive and participate in a series of chain reactions to become stable by acquiring electrons from nucleic acids, lipids, proteins, carbohydrates or other nearby molecules. The damage caused by free radicals to biological molecules, especially DNA, lipids and proteins, have been implicated in many human disease states (Halliwell, 1994). ROS can react with nitric oxide (NO), a free radical molecule, to generate highly reactive nitrogen species (RNS), such as peroxynitrite, nitrogen dioxide and dinitrogen trioxide, which exert deleterious effects on cell function referred to collectively as nitrative or nitrosative stress (Squadrito and Pryor, 1998).

Humans have developed complex antioxidant systems to counteract ROS/RNS and reduce their damage. These antioxidant systems include large molecule enzymes such as superoxide dismutase (SOD), catalase, and glutathione peroxidase (GSH-P_x); macromolecules such as albumin, transferrin, myoglobin, and ferritin; and a group of small molecule antioxidants, including ascorbic acid, α-tocopherol, ß-carotene, ubiquinol-10, reduced glutathione (GSH), methionine, uric acid, and bilirubin (Yu, 1994).

Oxidative/nitrative stress occurs when the generation of ROS/RNS exceeds the human system's ability to neutralize and eliminate them. This disturbed balance can be a consequence of excess production of ROS/RNS or depletion of antioxidants due to various nutritional insults or both, resulting in damage to proteins, lipids, and DNA (Gutteridge, 1995). The reference to either oxidative or nitrative stress will be made to clarify the role each has to play as biomarkers of human disease.

2.2.1.2. Oxidative stress processes: lipid peroxidation

Lipid peroxidation is a normal process that takes place continuously at low levels in humans (Kappus, 1985, Little and Gladen, 1999). Increased levels of lipid peroxides may result from increased production, as well as decreased antioxidant activities.

Peroxidation reactions are toxic to cells and cell membranes and are therefore normally controlled by counteracting biological mechanisms (Little and Gladen, 1999). Uncontrolled lipid peroxidation, which can result in cellular dysfunction and damage, has been associated with pregnancy-related complications (Hubel *et al.*, 1989).

The process of lipid peroxidation begins when free radicals attack polyunsaturated fatty acids or cholesterol in membranes or lipoproteins (Yagi, 1994). After the reactive radical removes hydrogen from the polyunsaturated fatty acid chain, the resulting carbon radical reacts with oxygen and this product then attacks adjacent fatty acid chains to generate a new carbon radical, causing a chain reaction to occur. The fatty acids in the resulting lipid peroxidation chain are converted to the primary products of lipid peroxidation, the highly reactive and unstable lipid hydroperoxides, and to secondary metabolites (Halliwell and Gutteridge, 1990, Yagi, 1994, Little and Gladen, 1999).

McConathy *et al.* (1981) studied the lipid content of human amniotic fluid and concluded that the predominant lipid species in amniotic fluid were free fatty acids. They also concluded that the major form of esterified fatty acids was phospholipid. The dissimilarities of total fatty acid composition of amniotic fluid to either cord serum or neonatal urine indicates that other tissue sources for fatty acids are found in amniotic fluid.

2.2.1.3. Markers of oxidative stress

Lipid peroxidation is a well-established indicator of oxidative stress in cells and tissues (Gutteridge, 1995). Lipid peroxides are readily broken down and generate a mixture of end-products which include alcohols, ketones, aldehydes, and ethers (Hubel *et al.*, 1989). Assays of potential use to quantify lipid peroxidation include the following: conjugated dienes, lipid hydroperoxides, thiobarbituric acid-reactive substances (TBARS) or malodialdehyde (MDA), alkanes, F_2-isprostanes, total plasma antioxidant capacity, or plasma glutathione levels (Dotan *et al.*, 2004).

TBARS:

The TBARS method is the most commonly used method to assess lipid peroxidation and measures one of the breakdown products of lipid peroxidation, MDA (Jardine *et al.*, 2002). TBARS quantification in body fluids may be inaccurate due to several factors which include the fact that substances other than MDA form chromogens

at 532 nm, MDA is formed during the assay procedure, antioxidants can interfere with the assay, and MDA can be derived from the diet (Wong *et al.,* 1987). However, TBARS in addition to MDA, 4-hydroxy-2-nonenal (HNE) and other aldehydes can be quantified by high performance liquid chromatography (HPLC) or gas chromatograph/mass spectrometry (GC/MS) in order to overcome the lack of specificity, sensitivity and reproducibility associated with the assay. The TBARS assay remains important because it can be easily performed without complicated instrumentation and is widely available.

F_2-isoprostanes:

Isoprostanes, a series of prostaglandin-like products formed in vivo by free radical-catalyzed nonenzymatic peroxidation of arachidonic acid independent of the cyclooxygenase enzyme, have emerged as novel markers of lipid peroxidation (Roberts and Morrow, 2000). Supporting evidence has evolved suggesting that the quantification of these compounds may be an important step towards assessing the status of oxidative stress *in vivo* (Roberts and Morrow, 2000, Longini *et al.,* 2005) since most assays have been more accurate when quantifying lipid peroxidation *in vitro* than *in vivo.* In plasma, isoprostanes exist in the free and phospholipid-bound state (Barden *et al.*, 1996). 8-isoprostane (F_2-IP) is the most abundant of the isoprostane products of free radical oxidation and exerts strong biological activity. High concentrations of F_2-IPs have been reported in plasma and placental tissues of pregnant women with different pathological pregnancies (Walsh *et al.,* 2000, McKinney *et al.,* 2000, Coughlan *et al.,* 2004). The measurement of F_2-IPs as reliable indicators of oxidative stress has several favorable attributes including high specificity, stability of the compounds, levels which are detectable in all normal biological fluids and tissues, and their levels are not affected by lipid content of the diet (Roberts and Morrow, 2000). Hence, the ideal assay for the measurement of lipid peroxidation for the assessment of oxidative injury becomes one which is accurate, specific, sensitive and where compounds quantified are stable (Morrow *et al.,* 1999).

2.2.1.4. Oxidative stress in normal human pregnancy

Pregnancy represents a state of oxidative stress resulting from increased mitochondrial activity and production of ROS (Agarwal *et al.*, 2005). Both lipid

peroxidation and antioxidation reactions are enhanced during pregnancy (Wang *et al.*, 1991a). Various studies have reported higher levels of lipid peroxidation markers such as TBARS (Iioka, 1994, Takehara *et al.,* 1990), lipid hydroperoxides (Gladen *et al.,* 1999) and conjugated dienes (Uotila *et al.,* 1991) in pregnant women compared with nonpregnant women.

During normal pregnancy, the levels of both lipoproteins and lipid peroxidation products increase with gestational age. Lipid peroxidation markers have been shown to increase during the first and second trimesters while a decline to baseline levels was observed during the third trimester (Little and Gladen, 1999). Levels of lipid peroxide markers across gestation in normal pregnancies have been reviewed and summarized by Little and Gladen (1999) (shown in Table 1). Most of the studies cited have relied on the TBARS method in plasma/sera (Sane *et al.*, 1989, Davidge *et al.,* 1992, Carone *et al.,* 1993, Iioka, 1994). By the second trimester, values of lipid peroxides increased by 10 to 50 percent over first trimester values, while third trimester sometimes but not always declined.

Table 1. Lipid peroxide marker levels over gestation in women with uncomplicated pregnancy, and comparison to nonpregnant women: ratio of marker before and during pregnancy to marker in first trimester

Figure taken from: Little RE and Gladen BC. Levels of lipid peroxides in uncomplicated pregnancy: a review of the literature. *Reprod Toxicol* 1999;13:347-52.

Author	N_1/N_2[a]	Method	Nonpregnant	1st trimester	2nd trimester	3rd trimester
Longitudinal						
Gladen (25)	21/28	Lipid hydroperoxides	0.69	1.00	1.12	0.88
Laverro (33)	20/10	TBA	1.01	1.00	1.14	1.46
Uotila (43)	—/10	TBA	—	1.00	0.71	0.50
		Conjugated dienes	—	1.00	1.46	1.08
Carone (27)	10/10	TBA	1.02	1.00	0.94	1.40
Cross-sectional						
Iioka (28)[b]	20/20	TBA	0.90	1.00	1.61	2.47
Ishihara (30)	22/58	TBA	1.02	1.00	1.25	1.41
Rebelo (36)	11/40	TBA	0.59	1.00	1.52	1.44
Takehara (37)[c]	11/26	TBA	1.19	1.00	1.50	1.92
Wang (39)	30/10	TBA	0.72	1.00	—	1.03
Twardowska (38)[d]	20/14	TBA	0.84	1.00	1.16	1.27

[a] N_1 is number of nonpregnant women; N_2 is number of pregnant women in cohort for longitudinal studies, and largest number of pregnant women measured in any trimester in cross-sectional studies.

[b] Study description does not specify if design is longitudinal or cross-sectional.

[c] Data reported as first trimester is from 5–9 weeks, second trimester is 20–29 weeks, and third trimester is at delivery.

[d] First trimester value estimated from nonpregnant and second trimester values.

Lipid peroxide levels are also induced in placental tissue and change over the course of gestation (Walsh and Wang, 1993a). While one study (Sekiba and Yoshioka, 1979) found that the peak production in vitro is achieved early in the placenta around the third month and diminished towards the end of pregnancy, another study (Diamant *et al.*, 1980) found that placental lipid peroxide formation was higher in third trimester than first trimester placentas. The decreasing levels of lipid peroxides during gestation reported in the first study was explained by increased placental activities of SOD and catalase with advancing gestation (Sekiba and Yoshioka, 1979, Takehara *et al.*, 1990). In contrast, Wang *et al.* (1991a) observed an increase in maternal serum concentrations of lipid peroxides in the third trimester which was counteracted by an increase in vitamin E during pregnancy compared to nonpregnancy. The increase in the ratio of vitamin E to lipid peroxides may be due to the protective biological effect of antioxidant capacity as gestation advances in normal pregnant women.

The relationship between maternal and fetal concentrations of lipid peroxides in term pregnancy before the onset of labor was examined by Rogers *et al.* (1999). Maternal plasma MDA was double that of cord blood whereas maternal organic hydroperoxide (OHP) was only 18% higher. In the absence of labor, free radical formation is either

lower in fetal than in maternal tissues in which the placenta maintains a significant gradient between the two circulations, or the placenta actively removes peroxides from the fetal circulation. The much lower transplacental gradient for OHP suggests that this lipid peroxidation product is not transferred across the placenta to the same extent as MDA.

Rogers *et al.* (1999) examined the relationships between maternal and fetal concentrations of lipid peroxides in term pregnancies before the onset of labor. They found a strong correlation between maternal and fetal concentrations of both organic hydroperoxides (products of lipid peroxidation) and MDA, suggesting a possible mechanism of free radical scavenging. There was a significant contribution of cord arterial MDA to variance in maternal MDA concentrations, which would suggest that trans-placental transport occurs. Comporti *et al.* (2004) found that plasma isoprostanes were significantly increased in plasma of neonates compared to adults. The study also strengthened the notion that some form of lipid peroxidation is active in the fetus. Most of the available literature, however, is consistent with the release of lipid peroxidation products into the maternal side to a much greater extent than the fetal side (Rogers *et al.*, 1999, Walsh *et al.*, 2000).

2.2.1.5. Oxidative stress in complicated pregnancies

2.2.1.5.1. Pregnancy-induced hypertension (PIH)

Hypertension is the most common medical complication of pregnancy and occurs in about 6-8% of all pregnancies (DeVoe and O'Shaughnessy, 1984, Chesley, 1984). It encompasses a group of disorders which include preeclampsia, eclampsia, latent or chronic essential hypertension, a variety of renal diseases, and transient (gestational) hypertension. Transient hypertension is defined as the acute onset of hypertension in pregnancy or the early puerperium without proteinuria or abnormal edema and resolving within 10 days after delivery. Chronic hypertension that had been latent prior to the pregnancy may also become evident during gestation. Pregnant women with latent chronic hypertension are at increased risk for stillbirth, neonatal death, and other fetal complications, but the risk is much lower than that of women with preeclampsia or eclampsia. Women with transient or latent chronic hypertension are also more likely to

develop chronic hypertension in later years (WHO, 1987, National High Blood Pressure Education Program Working Group, 1990, Beaulieu, 1994).

Preeclampsia, occurring in about 2.6 % of all pregnancies, is the most dangerous of these hypertensive disorders. Although definitions differ, the classification of preeclampsia is generally described as acute hypertension (blood pressure greater than 140 mm Hg systolic or 90 mm Hg diastolic) presenting after the 20th week of gestation, accompanied by abnormal edema and/or proteinuria, or both (WHO 1987, National High Blood Pressure Education Program Working Group, 1990). This definition generally guides clinical management rather than epidiomological purposes since it is less stringent than that used for research purposes.

Women with preeclampsia are at increased risk for such complications as abruptio placentae, acute renal failure, cerebral hemorrhage, disseminated intravascular coagulation, pulmonary edema, circulatory collapse, and eclampsia (Canadian Task Force on the Periodic Health Examination, 1994). The fetus may become hypoxic, increasing its risk of LBW, premature delivery, or perinatal death. Individuals at increased risk of developing preeclampsia and eclampsia include primigravidas and women with multiple gestations, molar pregnancy or fetal hydrops, chronic hypertension or diabetes, or a personal or family history of eclampsia or preeclampsia (Cunningham and Lindheimer, 1992, Roberts and Redman, 1993, Cunningham *et al.,* 1993).

Elevated lipid peroxidation in women with preeclampsia was first substantiated by identifying increased serum levels of MDA. Numerous other studies have been performed which confirmed that higher levels of sera/plasma lipid peroxidation products, usually measured as TBARS, were present in preeclamptic women versus their normotensive pregnant controls. Pasaoglu *et al.* (2004) found that plasma MDA and uric acid concentrations were higher in preeclamptic (4.4 +/- 1.7 nmol/ml and 0.45 +/- 0.11 mmol/liter respectively) and eclamptic (5.8 +/- 1.9 nmol/ml and 0.47 +/- 0.12 mmol/liter) groups compared with control group (3.0 +/- 1.3 nmol/ml; 0.35 +/- 0.06 mmol/liter). Erythrocyte MDA concentrations were higher only in the eclamptic group compared to controls. Hence, the balance between lipid peroxide products and antioxidants present are important factors in the pathogenesis of preeclampsia and eclampsia.

The role of oxidative stress and endothelial cell dysfunction has been extensively reviewed in the pathogenesis of preeclampsia (Hubel, 1999, Poston and Chappell, 2001). Lipid peroxidation has been linked with the induction of endothelial cell dysfunction which leads to the clinical manifestation of preeclampsia (Hubel *et al.,* 1989, Walsh, 1994). Lipid peroxides vasoconstrict the human placenta by stimulating thromboxane production and inhibiting prostacyclin synthesis. Since circulating peroxidation products may originate from the placenta (Walsh and Wang, 1993a, Hubel, 1999), it is believed that abnormal placentation is involved in the induction of both preeclampsia and IUGR.

Sane *et al.* (1989) assessed the serum levels of lipoperoxides in forty pregnancy induced hypertension (PIH) subjects, before and after antihypertensive drug therapy, using full term normotensive patients as controls. The PIH subjects demonstrated highly significant elevated serum levels of lipoperoxides (mean 3.60 nmol/ml) in comparison with full term normotensive subjects (mean 2.15 nmol/ml). The PIH subjects showed a highly significant fall in serum lipoperoxide following antihypertensive drug therapy postpartum; however these levels were still significantly elevated when compared to those of full term normotensives at post-partum, suggesting that monitoring serum lipoperoxide levels can serve as one of the useful parameters in PIH subjects.

Placental tissue levels and production rates of lipid peroxides are significantly higher in placentas obtained from women with preeclampsia than from women with normal pregnancies (Wang *et al.*, 1992, Walsh *et al.*, 1992). The rate of lipid peroxidation in the placenta has also been reported to be abnormally high in preeclampsia (Wang *et al.,* 1992, Gratacos *et al.,* 1998, Mutlu-Turkoglu *et al.,* 1998, Little and Gladen, 1999). One possible explanation for this is that there are insufficient levels of antioxidants (Wang and Walsh, 1996). In a study of women undergoing cesarean section, higher concentrations of lipid hydroperoxides, phospholipids, and cholesterol were detected in deciduas basilis tissues from women with preeclampsia as compared to tissues from normal controls (Staff *et al.*, 1999a).

The potent direct and indirect vasoconstrictor effects of 8-isoprostane may contribute to the pathogenesis of preeclampsia. Walsh *et al.* (2000) studied two markers of oxidative stress in placental tissue of preeclamptic and normal pregnancies and found that free and total isoprostane were significantly higher for preeclamptic placentas and

that MDA concentrations were highly correlated with those of isoprostane. They suggested that elevated levels of isoprostane in the placenta could contribute to placental vasoconstriction and its secretion into the maternal circulation could cause vasoconstriction in the maternal vascular beds. Barden *et al.* (1996) showed that women with preeclampsia had elevated free but not total plasma 8-isoprostane and lower urinary 8-isoprostane excretion compared with normal women. However, total plasma 8-isoprostane levels did fall significantly in the preeclamptic women post-partum, suggesting that they had relatively higher levels compared to their nonpregnant state. Another study by Staff *et al.* (1999b) revealed that the content of free 8-isoprostane but not total isoprostane, in deciduas from women with preeclampsia is nearly twice the concentrations of normal pregnancies.

2.2.1.5.2. Diabetes

Increased oxidative stress has been associated with diabetic states (Baynes, 1991). Evidence of the link between diabetes, especially type 2, and oxidative stress has been described in studies that have found increased biomarkers of oxygen radical damage as well as abnormalities in the antioxidant defense systems. In diabetic conditions, protein glycation and glucose autoxidation may lead to the generation of free radicals. The elevated levels of free radicals can react with proteins or lipids, hence promoting lipid peroxidation processes and resulting in oxidative damage (Baynes, 1991, Hunt *et al.*, 1991). Although the mechanism by which oxidative stress in increased in diabetes is not clear, there is considerable evidence that high blood glucose levels induce oxidative stress and decrease antioxidant defenses, leading to increased free radical formation and hence oxidative damage (Baynes, 1991, Hunt *et al.*, 1991, Giugliano *et al.*, 1996, Kamath *et al.*, 1998, Baynes and Thorpe, 1999). Santini *et al.* (1997) reported raised levels of conjugated dienes in Type 1 diabetes. They also found elevated levels of lipid peroxides and lowered total radical trapping capacity in this group. Plasma lipid peroxides were more than doubled in type 2 diabetic subjects compared with controls (Nourooz-Zadeh *et al.*, 1997). Serum levels of TBARS were significantly increased in both Type 1 and Type 2 diabetics (Griesmacher *et al.*, 1995). In addition, 8-isoprostane was shown to be both an oxidative stress indicator and teratogenic agent in rat embryos in vitro, while the

administration of antioxidant agents normalized all morphological and biochemical variables (Wentzel and Eriksson, 2002). Urinary and plasma concentrations of 8-isoprostane were elevated in individuals with type 2 diabetes (Gopaul *et al.,* 1995, Davi *et al.*, 1999, Deveraj *et al.*, 2001).

Experimental diabetes can be induced in rodents by feeding alloxan or streptozotocin (STZ), which both work by generating ROS. Elevated isoprostanes were found in pregnant rats with STZ-induced diabetes (Gerber *et al.*, 2000). Feillet-Coudray *et al.* (1999) showed that liver TBARS levels in STZ-induced diabetic rats were significantly increased after 4 weeks of diabetes despite increased vitamin E liver content. However, plasma isoprostane concentrations were not modified at 1 week or 4 weeks after induction. Cederberg *et al.* (2001) found no significant differences in maternal plasma concentrations of 8-iso-PGF2α between pregnant diabetic rats and control rats; however, plasma protein carbonyl content, a widely used biomarker of severe oxidative protein damage, was increased. There was no difference in amniotic fluid concentrations of 8-iso-PGF2α between non-diabetic and diabetic rats; however both the isoprostane and the prostaglandin metabolite concentration tended to be higher in amniotic fluid from resorbed fetuses (dead offspring) indicating increased lipid peroxidation in this group.

Limited data are available regarding the role of oxidative stress in GDM, a disease of similar pathology. GDM has been shown to induce a condition of oxidative stress, where lipid and protein oxidative damage occurs (Kamath *et al.*, 1998). The placenta may protect the fetus from the adverse diabetic environment; however disturbances in placental function may also impair fetal outcome (Diamant, 1991). 8-isoprostane is capable of inducing vasoconstriction of the placenta (Kwek *et al.,* 2001). Placental release of 8-isoprostane was 2-fold greater from women with GDM compared to healthy pregnant women, suggesting that GDM mothers have an underlying placental lipid peroxidation as a consequence of experiencing increased oxidative stress (Coughlan *et al.,* 2004). Djordjevic *et al.* (2004) investigated oxidative stress parameters in 90 patients subdivided into 3 groups: pregnant women with Type 1 diabetes mellitus, healthy pregnant women and non-pregnant women. Diabetic control was monitored by fasting blood glucose and glycosylated hemoglobin (HbA1c). TBARS concentration increased significantly in pregnant women when compared with non-pregnant controls, as well as in

pregnant diabetics compared with healthy pregnant women. High lipid peroxidation products were seen in pregnant women with insulin-dependent diabetes mellitus at each trimester when compared to healthy pregnant women (Carone *et al.*, 1993). Erythrocyte MDA levels were significantly elevated in babies born to mothers with GDM when compared to controls and there was a significant correlation between glycated haemoglobin levels and MDA levels of the GDM mothers (Kamath *et al.*, 1998).

2.2.1.5.3. Other outcome measures

In addition to maternal diseases, other conditions including smoking, obesity, delivery method, and low Apgar scores, may contribute to oxidative stress and hence were explored in the literature. There is some literature indicating that labor increases oxidative stress (Wang *et al.*, 1996, Buonocore *et al.*, 2000); however, information on the effect of the delivery method on oxidant and antioxidant systems is not decisive yet. Woods *et al.* (2002) showed that mean vitamin C concentrations in amniotic fluid of women undergoing labor and vaginal delivery were 29% lower than values obtained from subjects undergoing elective repeat caesarean section without labor.

Rao *et al.* (2003) also demonstrated that various obstetric complications can create oxidative stress in both mother and fetus. Lipid peroxidation, measured through MDA levels, was significantly increased in erythrocytes of fetuses born after premature rupture of membranes (PROM) as compared to healthy fetuses. Elevated lipid peroxidation in maternal and cord blood was also reported by Yin *et al.* (1995). Lipid peroxidation was also higher in erythrocytes of mothers with prolonged second stage of labour and those who delivered by lower segment caesarean section as compared to controls. In contrast, Comporti *et al.* (2004) reported that there were no significant differences between the plasma F_2-IPs concentrations for term and preterm newborns with vaginal delivery and those with cesarean section, suggesting that the delivery method may not have an impact on the increased oxidative stress. They also found a significant negative correlation between the plasma isoprostane levels and the APGAR score, reflecting an increased stress at birth in infants with lower APGAR scores. Increased ROS production due to hypoxia (Buonocore *et al.*, 2000) may explain this finding.

The relationship between increased lipid peroxidaton and smoking is well established (Morrow *et al.,* 1995, Zhou *et al.*, 2000). Hulea *et al.* (1995) demonstrated that a prooxidant/antioxidant imbalance exists in the blood of smokers; they found that in smokers between 46 to 80 years but not in the 18 to 45 age group, the changes in plasma prooxidant parameters such as lipid peroxides and leukocyte activation as well as antioxidant parameters of thiol concentrations and total antioxidant capacity were significantly different from those of the age-matched controls. They also noted increased activity of both antioxidant erythrocyte enzymes, GSH-Px and SOD, in the 18 to 45 age group and decreased activity in the 46 to 80 age group. Smoking is also known to decrease levels of antioxidant nutrients in plasma, specifically ascorbic acid and beta carotene (Palan *et al.,* 1989, Basu *et al.,* 1990).

Evidence suggests that a combination of sources of oxidative stress ensues in obesity, including hyperglycemia, hyperleptinemia, increased tissue lipid levels, inadequate antioxidant defenses, increased rates of free radical formation, and chronic inflammation (Vincent and Taylor, 2006). In order to investigate the relationship between lipid peroxidation and obesity, Furukawa *et al.* (2004) measured plasma TBARS and urinary 8-epi-prostaglandin-F2α (8-epi-PGF2α or F2-IP) in non-diabetic human subjects and found them to be significantly correlated with BMI and waist circumference. They also found significant inverse correlations between plasma adiponectin and plasma TBARS as well as urinary F2-IP. This may imply that fat accumulation alone could increase systemic oxidative stress independent of hyperglycemia. Keaney *et al.* (2003) also suggested a correlation between systemic oxidative stress and BMI as they found a positive correlation between urinary creatinine-indexed F2-IP and BMI. Olusi (2002) found decreased concentrations of plasma MDA in subjects with a healthy BMI than in those with BMI above 40 kg/m^2. On the other hand, subjects with a healthy BMI had higher erythrocyte CUZn-SOD and GPX than those with BMI above 40 kg/m^2.

2.2.1.6. Oxidative stress in fetal growth

The conditions associated with adverse fetal growth have often been attributed to oxidative stress (Luo *et al.,* 2006). The oxidative stress programming hypothesis is demonstrated in Figure 1. The concept of oxidative stress programming may arise

through the role of oxidative stress in modulating gene expression directly or indirectly through the influence of oxidized molecules. Experimental studies have established the role of redox balance in modulating gene expression, and recent studies indicate that both insulin and blood pressure may be sensitive targets to oxidative stress programming. Pregnancy is a period whereby oxidative stress is amenable to change; hence, measures that can remove this nutritional insult early on could be instrumental in preventing the increasing epidemic of the metabolic syndrome, type 2 diabetes, and cardiovascular disease.

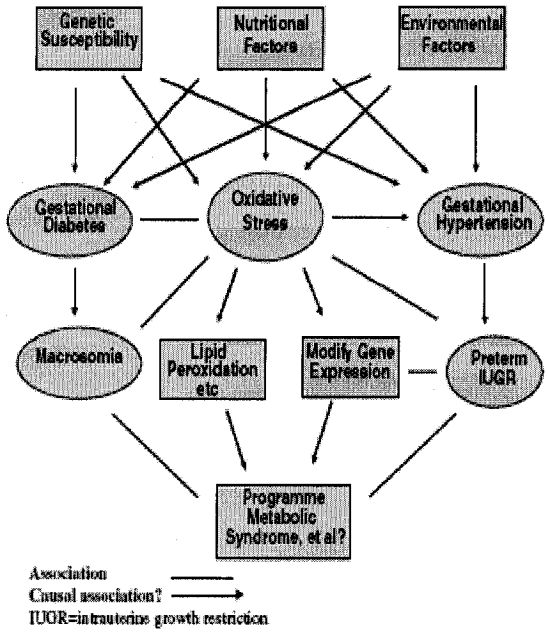

Figure 1 Oxidative stress may be the key link between adverse insults (associated with preterm birth or adverse fetal growth) and fetal or developmental programming of the metabolic syndrome, type 2 diabetes or cardiovascular disease through directly modulating gene expression and/or the indirect effects of oxidized lipids or other molecules.

Figure taken from: Luo ZC, Fraser WD, Julien P, Deal CL, Audibert F, Smith GN, Xiong X, Walker M. Tracing the origins of "fetal origins" of adult diseases: programming by oxidative stress? *Medical Hypotheses* 2006;66(1):38-44.

An increase in oxidative stress and in placental levels of redox-related molecules has been reported in preeclampsia and FGR (Takagi *et al.,* 2004). Preeclampsia, with or without IUGR, differs from IUGR present alone in that the pathophysiological changes are extended into the maternal vasculature. Using immunohistochemistry and Western blotting, the researchers investigated oxidative stress and redox-related molecules in

placenta of preeclamptic patients, IUGR, preeclampsia + IUGR, and normal pregnancy. The level of hydroxy-2′-deoxyguanosine (8-OHdG) was significantly higher in preeclampsia with or without IUGR, while thioredoxin (TRX) was higher in all three treatment groups. The levels of 4-hydroxy-enal (4-HNE) did not differ between the groups, while redox factor-1 (ref-1) was enhanced in complicated vs. normal pregnancy (Takagi *et al.,* 2004). These results suggest that oxidative DNA damage is higher in IUGR and that redox function is increased in both preeclampsia and in IUGR.

Various parameters reflecting oxidative damage including MDA, lipid hydroperoxides and conjugated dienes were significantly increased in mothers with IUGR compared to normal pregnancies (Karowicz-Bilinska *et al.,* 2002, 2004). Kamath *et al.* (2006) showed that lipid peroxidation was significantly increased as a result of elevated MDA levels in erythrocytes of both mothers and newborns with IUGR. However, Pasaoglu *et al.* (2003) found no significant difference in plasma MDA levels between IUGR and control groups. This may be related to the premises that both NO and MDA levels are implicated in the pathogenesis of eclampsia; however with regards to IUGR pathogenesis, NO and not lipid peroxidation is involved (Pasaoglu *et al.*, 2003).

Serum MDA was higher in cord blood of term SGA newborn infants born to undernourished mothers compared to term AGA infants born to healthy mothers, suggesting that intrauterine malnutrition is associated with significant oxidative stress in infants with IUGR (Gupta *et al.*, 2004). Comporti *et al.* (2004) discovered that placental total (sum of free plus esterified) F_2-isoprostanes were significantly higher in preterm deliveries than in term deliveries and that plasma isoprostane levels were inversely correlated with gestational age. Another study which examined the biomarker 8-isoprostane in serum, found elevated levels in women diagnosed with IUGR (Karowicz-Bilinska *et al.,* 2003a).

Very few studies have measured lipid peroxidation products in amniotic fluid. Bazowska *et al.* (1994) compared MDA concentrations in amniotic fluid collected after parturition in 56 pregnant women who were diagnosed with IUGR with those of 35 uneventful pregnancies. They found that the MDA concentration in amniotic fluid was almost three times higher in pregnancies complicated by IUGR than in normal pregnancy

(0.785 mmol/l and 0.268 mmol/l, respectively) and concluded that determination of MDA can be used as a biochemical diagnostic indicator of IUGR.

Longini *et al.* (2005) found elevated isoprostane concentrations in the amniotic fluid of FGR pregnancies collected at 15 to 18 weeks of gestation. Since 17 out of the 37 pregnancies with FGR were born AGA, the researchers also investigated the reliability of isoprostane markers in amniotic fluid in second trimester to predict the delivery of AGA or SGA infants. However, there were no statistical differences in isoprostane levels between AGA and SGA newborns within the FGR group. The relative risk index of being born below the 25th percentile was 8.05 times greater in the FGR group vs. normal fetal growth group. The results may suggest that the assay of isoprostanes in amniotic fluid may represent an assessment of fetal oxidative stress and that this oxidative stress may stem at an early stage of fetal life (Longini *et al.,* 2005).

2.2.2. Nitric oxide (NO): friend or foe?

2.2.2.1. Definition and Synthesis

Nitric oxide (NO) is a free radical molecule that has been identified as acting as a neurotransmitter, paracrine substance, and hormone (Buhimschi *et al.,* 1998). NO is now known to play an important role in the biology, physiology and pathophysiology of reproductive systems (McCann *et al.*, 1996, Gregg, 2003). Through its various sites and modes of production, NO commands a regulatory network of actions in both normal and abnormal pregnancy (Rosselli *et al.*, 1998, Buhimschi *et al.*, 1998). Some of the effects of NO have been well established such as its role in maintaining uterine quiescence, its vasodilatory function in impaired perfusion states such as preeclampsia, and its production in response to infectious metabolic products (Miller *et al.*, 1996, von Mandach *et al.*, 2003). Alterations in the NO pathway during pregnancy may therefore play a role in both the physiological changes of advancing gestation, and may contribute to the pathophysiology of preeclampsia and fetal growth retardation (Sooranna *et al.*, 1995). However, fewer studies have identified the origins of NO and its differential role among the maternal and fetal compartments. More specifically, evidence regarding the NO system in amniotic fluid is limited and inconclusive. Before studying NO in amniotic fluid, it is important to define its biological and physiological role.

NO is synthesized from L-arginine by the action of NO synthase (NOS), an enzyme that exists in three isoforms. Brain NOS (bNOS) or neuronal NOS (nNOS or NOSI) and endothelial NOS (eNOS or NOS III) are responsible for the continual basal release of NO, while the third isoform, inducible NOS (iNOS or NOS II), is expressed only in response to inflammatory cytokines and lipopolysaccharides (LPS). Functionally, the most important difference between NOS enzymes is that constitutive NOS enzymes are low output (picomolar) while iNOS is a high output enzyme which produces large quantities of NO (nanomolar) in short periods (McDonald *et al.,* 1996, Rosselli *et al.*, 1998).

Many researchers have used NO inhibitors in animal models to study the pathophysiology of decreased NO synthesis, which has allowed further investigation of the effects of NO deficiency on the fetus. NOS activity is dependent on substrate availability and five additional co-factors (FMN, FAD, Heme, calmodulin and tetrahydrobiopterin). The availability of these factors determines the cellular rates of NO synthesis. L-arginine is the only physiological nitrogen donor for NOS-catalyzed reactions and availability of this essential substrate can determine rates of NO generation. Competitive inhibition of arginine uptake by L-lysine, L-ornithine (naturally occurring amino acid) and nitro-L-arginine methyl ester (L-NAME) or N^g-monomethyl-L-arginine (L-NMMA, arginine analogues) results in decreased NO synthesis; hence, these substances have been used in restricted NO models (Rosselli *et al.*, 1998).

2.2.2.2. NO System in Pregnancy

The formation of the placenta during early pregnancy involves angiogenesis in maternal and fetal placental tissues and is accompanied by a marked increase in uterine and umbilical blood flows. The large increase in transplacental exchange, which supports the exponential growth of the fetus during the second half of gestation, depends heavily on the dramatic growth of the placental vascular beds and the subsequent large increases in uterine and umbilical blood flows. Increased uterine vascular resistance and reduced uterine blood flow have been associated with IUGR. Hence, factors that influence placental vascular development and function will have a dramatic impact on fetal growth and development; thereby, a close relationship with neonatal survival, growth and weight

is expected. An involvement of NO in the vascular angiogenic activity of the fetoplacental unit has been suggested (Reynolds and Redmer, 2001, Tranquilli *et al.*, 2003).

Angiogenesis, or the formation of vascular beds, is a major component of the increase in placental blood flow throughout gestation (Reynolds and Redmer, 2001). *In vitro* and *in vivo* studies have shown that NO stimulates endothelial cell migration, proliferation, and differentiation into capillaries (Ziche *et al.*, 1994, Lee *et al.*, 2000). Most of the evidence suggesting that NO is a pro-angiogenic mediator is based upon data showing that growth factors such as the vascular endothelial growth factor (VEGF) upregulate the expression and activity of NOS.

Different NOS isoforms may play different roles throughout pregnancy (Marinoni *et al.*, 2000). Namely, iNOS seems to be the isoform responsible for the large amount of NO produced within the uterus and placenta during pregnancy, while eNOS produces NO in placental vascular endothelium with the primary purpose of promoting vasodilation and increasing blood flow. During conditions in which perfusion is impaired by hypertensive disease in pregnancy, compensatory production of NO by eNOS in the placental vascular bed promotes vasodilation and hence enhances perfusion (von Mandach *et al.*, 2003). Myatt *et al.* (1997b) demonstrated that the expression of eNOS, and hence NO synthesis, is increased in fetal-placental vasculature obtained from patients suffering from preeclampsia with or without IUGR, possibly as an adaptive response to the increased vascular resistance, poor perfusion and hypoxia. Due to the vasodilator effects of NO, an impairment in NO production has been proposed as a possible mechanism for reducing the fetoplacental circulation (Sooranna *et al.*, 1995, Myatt *et al.*, 1997b).

Variations in the response to NO during gestation have been well established. The relaxing effect of NO is enhanced during most of gestation until term or preterm labour (Izumi *et al.*, 1993; Natuzzi *et al.*, 1993; Weiner *et al.*, 1994). The NO system is up-regulated during pregnancy and inhibits uterine contractility until term; however, it decreases once labour begins. This suggests that intrinsic, autocrine NO activity in the uterus may contribute to the maintenance of uterine quiescence during pregnancy and its withdrawal prior to term may prompt parturition (Buhimschi *et al.*, 1998). The

expression of myometrial iNOS in both rats and humans has been shown to be up-regulated in pregnancy as compared to non-pregnant subjects and to fall at term (Riemer *et al.*, 1997, Bansal *et al.*, 1997).

Various studies have reported the increase in NO production in the fetoplacental unit during IUGR when compared to normal pregnancy concentrations (Morris *et al.*, 1995a, Lyall *et al.*, 1996, DiIorio *et al.*, 1997). Placental NO was found to be significantly associated with uteroplacental blood flow and has gained importance as a factor in maintaining adequate uteroplacental perfusion (Sooranna *et al.*, 1995). Compromised NO production in the uterine placental bed may play a crucial role in the impaired uteroplacental blood flow of pathologic pregnancies such as preeclampsia and IUGR (Seligman *et al.*, 1994, Moriss *et al.*, 1996, DiIorio *et al.*, 1997, Tranquilli *et al.*, 2003). Moreover, elevated vasodilatory NO production in utero was found to persist during early neonatal life in small-for-gestational age infants, where urinary nitrate + nitrate excretion increased from 51 ± 12 at 0-6 h to 56 ± 17 mmol NOx/mol creatinine at 24 hr compared to an increase of 26 ± 3 at 0-6hr to 56 ± 17 mmol NOx/mol creatinine at 168 h in AGA (Wijnberger *et al.*, 2001). On the other hand, Hata *et al.* (1998) detected that maternal and fetal sera NO metabolites (6.91 ± 1.27 μM and 7.54 ± 1.09 μM respectively) at birth in SGA infants were significantly lower than those (11.69 ± 1.33 μM and 11.24 ± 1.08 μM respectively) in AGA infants. In preeclamptic pregnancies, NO metabolite levels in blood samples from the fetoplacental circulation were negatively correlated with gestational age and birth weight, representing a compensatory mechanism to offset the pathologic effects of impaired perfusion (Norris *et al.*, 1999).

2.2.2.3. Reactive nitrogen species (RNS) and nitrative stress

The nitric oxide molecule is a free radical, which makes it highly reactive causing it to react readily with other molecules to form a stable product (Violi *et al.*, 1999). The major end-products of NO metabolism are nitrite and nitrate. During metabolism to these end-products, NO can undergo a series of reactions that generate RNS. When RNS levels exceed the cellular antioxidant capacity, oxidative/nitrative stress occurs (Squadrito and Pryor, 1998). The influence of NO in oxidative/nitrative stress is a function of the ROS or antioxidants present, the relative concentrations of reactants and substrates and rate

constants; hence, NO shows both antioxidant and prooxidant effects (Bloodsworth *et al.,* 2000). However, NO production in biological systems also contributes to normal physiological processes and is involved in fetal growth. Thus, both an impairment in production as well as overproduction may be associated with pathologies during pregnancy.

On the one hand, NO is strongly implicated in the vasodilation of the maternal systemic circulation and regulation of uterine and feto-placental blood flow (Myatt *et al.,* 1991, Conrad *et al.,* 1993, Lyall and Greer, 1996). The human fetal-placental vasculature lacks autonomic innervation, and hence must be regulated by autocrine/paracrine agents such as the NO radical. NO maintains low resistance and reduces the action of vasoconstrictors in the fetal-placental vasculature (Gude *et al.,* 1990, Myatt *et al.,* 1992). Hence, an impairment in NO production can reduce fetoplacental circulation in pregnancies and be associated with higher vascular resistance and abnormal pregnancy outcomes such as preeclampsia and IUGR (Rutherford *et al.,* 1995, Sladek *et al.,* 1997, Norris *et al.,* 1999); however, this is still somewhat controversial.

On the other hand, the interaction of NO with superoxide anion causes its inactivation and yields peroxynitrite anion, a powerful oxidant (Myatt *et al.,* 2000). The activity of NO is prolonged in the presence of SOD, which removes superoxide; however, when superoxide and NO concentrations are increased, NO outcompetes SOD for superoxide resulting in the formation of peroxynitrite, $ONOO^-$ (Van Der Vliet *et al.,* 1994). The balance between superoxide and NO may determine whether their combined release results in physiological effects, when NO is present in amounts greater than superoxide, or pathologic effects, when superoxide is present in amounts greater than NO (Holcberg *et al.,* 1995). Peroxynitrite is known to alter cellular functions in many ways: initiate lipid peroxidation, cause endothelial cell function, inhibit the mitochondrial electron transport system, and oxidize sulfhydral groups on proteins, thus altering their activity or disrupting signal transduction pathways (Myatt *et al.,* 1996, Kossenjans *et al.,* 2000). In vitro, peroxynitrite oxidizes a variety of classes of lipids such as purified fatty acids, neutral lipids, and phospholipids forming conjugated diene, MDA, lipid peroxide, lipid hydroxide, F_2-IP, and oxysterol products (O'Donnell *et al.,* 2001). Peroxynitrite can also nitrate many proteins, particularly tyrosine moieties (Myatt *et al.,* 2000). Kossenjans

et al. (2000) observed an increased expression of nitrotyrosine residues, which served as markers of peroxynitrite formation and action as well as indicators of nitrative stress, in the fetal vasculature and villous stroma of preeclamptic and diabetic placentas. Results also revealed that responses to both vasoconstrictors and vasodilators were significantly attenuated in diabetic and preeclamptic pregnancies as compared to controls (Kossenjans *et al.*, 2000). Nitrotyrosine residues have also been found in the fetal vasculature of preeclamptic and diabetic pregnancies, indicating nitrative stress (Myatt *et al.,* 1996, Lyall *et al.,* 1998, Roggensack *et al.,* 1999). Nitrative stress is seen in the placenta in preeclampsia and diabetes in association with altered placental function (Myatt *et al.*, 2004).

A deficiency in NO production or availability has been proposed as a contributing factor to the pathophysiology of preeclampsia (Chwalisz, 1996). However, there have been inconsistent reports about NO levels analyzed through circulating degradation products in preeclamptic pregnancies, including decreased (Seligman *et al.,* 1994), increased (Smarason *et al.,* 1997, Ranta *et al.*, 1999), or unchanged levels (Wang *et al.,* 1994, Davidge *et al.,* 1996). Ranta *et al.* (1999) suggested that NO production was increased in preeclampsia, possible due to a biological compensation of the vasoconstriction created in this disorder. Pasaoglu *et al.* (2004) found that plasma NO metabolites levels were higher in eclamptic group (35.7 +/- 16.5 μmol/l) but not in pre-eclamptic group (22.1 +/- 10.8 μmol/l) than control group (18.8 +/- 6.9 μmol/l). This may be due to the clinical progress of eclampsia being more severe than preeclampsia. These results were supported by Pathak *et al.* (1999) who showed that plasma nitrate and nitrite levels of sever preeclamptic patients were higher than mild preeclamptic patients. Shaamash *et al.* (2000) showed that placental nitric oxide synthesis activity and NO production were significantly increased in preeclampsia and eclampsia. Moreover, this increase was directly related to the severity of the disorder, suggesting that it represents a physiological adaptive response to overcome the increased placental vascular resistance. Roggensack *et al.* (1999) found that in the maternal vasculature of preeclamptic women, immunostaining for eNOS and nitrotyrosine was increased while that for SOD was decreased.

In contrast, Mutlu-Turkoglu *et al.* (1999) showed that the plasma levels of nitrite and nitrate were decreased in patients with preeclampsia. They also suggested that the increased levels of lipid peroxides in circulation may be caused by lowered NO synthesis. Morris *et al.* (1995a) investigated NOS activity in the placenta of ten normotensive, six preeclamptic, and eight growth retarded pregnancies and found significantly lower activities in preeclamptic and IUGR groups. Placental tissue levels in preeclampsia have been reported to be decreased in other studies (Brennecke *et al.*, 1997, Bocardo *et al.*, 1997). Hence, a deficiency of NO may lead to the impaired fetoplacental circulation seen in these complications of pregnancy. Finally, Silver *et al.* (1996) showed that circulating levels of nitrate and nitrite are not reduced in patients with severe preeclampsia compared with normotensive controls and that sera from these women do not suppress endothelial cell NO synthesis. The biosynthesis of NO during normal pregnancy and preeclampsia remains controversial, as reviewed by Sladek *et al.* (1997).

2.2.2.4. NO in Amniotic Fluid

The up-regulation of the NO pathway during pregnancy also contributes to the high rate of fetal swallowing in-utero, which is critical for amniotic fluid volume regulation in addition to the development of the fetal gastrointestinal tract. El-Haddad *et al.* (1999, 2001) demonstrated in the near-term ovine fetus model the contribution of NO, by the action of neuronal NOS, to the heightened level of spontaneous fetal swallowing behavior.

Similar roles for the NO system during normal pregnancy have been observed to occur if amniotic fluid and not plasma is the biological medium that is evaluated. Egberts *et al.* (1999) showed that NO-cGMP pathway is enhanced during pregnancy through changes in amniotic fluid concentration of total nitrite, cGMP, and these metabolites' ratios to dimethylarginine (DMA), an endogenous inhibitor of nitric oxide synthase. Thus, the increases of NO derivatives in amniotic fluid may be regulated with gestational age as a result of fetal growth and reduced inhibition of NO synthesis by DMA. In another study, NO metabolite levels in amniotic fluid were increased from early to late pregnancy and decreased slightly at term in both normal (n=103) and abnormal pregnancies (n=60), with the difference being strikingly higher between early and late pregnancy for abnormal

cases (von Mandach *et al.*, 2003). Given that intrauterine infection and inflammation seen in many abnormal subjects is typically associated with elevated amniotic fluid cytokine levels, cytokines induce the expression of iNOS, resulting in a heightened production of NO. This same study also revealed that higher NO concentrations in amniotic fluid than in maternal plasma or umbilical artery may indicate greater paracrine NOS expression in the fetal/placental tissue. This may reflect that the main source of NO comes from the fetal compartments and hence, that NO is a means by which the fetus maintains and regulates both pregnancy and onset of labor.

Other findings which demonstrate amniotic fluid NO's crucial role during pregnancy and labor include Morris *et al.* (1995b) who observed a high concentration of nitrite in amniotic fluid with no sequential change until 36 weeks gestation followed by an 80% significant reduction in nitrite concentration in late pregnancy. The decreased amniotic nitrite concentration after 37 weeks gestation may indicate that reduced placental derived NO contributes to uterine activity in late pregnancy, while the basal release during gestation maintains uterine quiescence.

2.2.2.5. Amniotic fluid NO, fetal growth and birth weight

Many animal studies have explored the role of the NO regulatory pathway during pregnancy, its association with fetal growth and development, and the effects of NOS inhibition on maternal and fetal health (Yallampalli *et al.*, 1993, Diket *et al.*, 1994, Salas *et al.*, 1995, Helmbrecht *et al.*, 1996). These experimental studies have demonstrated that elevated NO production may be partially responsible for regulating the vascular adaptation to pregnancy and that chronic reductions of NO synthesis in rats result in significant IUGR Yang *et al.* (1996) evaluated plasma and urine NO metabolites as well as amniotic fluid and found concentrations to be elevated in pregnant sheep in comparison to nonpregnant sheep. Diket *et al.* (1994) showed that the administration of an NOS inhibitor in the last trimester of pregnancy in rats results in marked growth retardation and hemorrhagic necrosis of hind limbs. The addition of an NO donor reduced the incidence of disruptions in a dose-dependent manner. Other rat models have shown the inhibition of NOS being closely tied with limb malformations (Pierce *et al.*, 1995). Hefler *et al.* (2001) showed that mice deficient in eNOS had abnormal prenatal

and postnatal development including FGR, reduced survival, and an increased rate of limb abnormalities. A prospective rat study by Witlin *et al.* (2002) examined the subsequent neonatal growth, fertility, and blood pressure for second generation rats following maternal exposure to N (G)-nitro-L- arginine methyl ester (L-NAME), an inhibitor of NOS. It was found that the prolonged in utero exposure to L-NAME in pregnant rats resulted in decreased neonatal weight, postnatal growth, and fertility of the offspring but no differences were found in blood pressure between treated and control groups.

Fewer human studies investigating the relationship between NO and fetal growth in amniotic fluid have been conducted. A retrospective study performed by Tranquilli *et al.* (2004) assessed NO and VEGF, a major angiogenic growth factor, on midtrimester amniotic fluid from seven women who subsequently had intrauterine fetal death before 20 weeks, and compared the results with 14 controls matched for age and gestation. Women with subsequent fetal death had lower amniotic NO and VEGF than women with a normal pregnancy. This suggests that an early reduction of both NO and VEGF may be responsible of an impaired placental vascular development and endothelial regulation that may lead to fetal death.

Another study by Tranquilli *et al.* (2003) retrospectively assessed amniotic fluid NO from second-trimester amniocentesis of 20 healthy normotensive women who subsequently developed IUGR and 20 controls matched for age and gestation. The same women were re-assessed in the third trimester after IUGR had developed and pair-matched with normal controls. At 16 weeks, NO levels in women with subsequent IUGR were significantly lower than controls, while at the 3rd trimester, in women with IUGR, NO levels were significantly higher than in normal pregnancies. A positive correlation was seen between amniotic NO values and time IUGR was diagnosed. Hence, low levels of NO during the early second trimester may represent an impaired stimulus to vascular formation and endothelial regulation, inducing placental disease and subsequent FGR. High levels of amniotic fluid NO during the third trimester, on the other hand, may represent a compensation factor for maintaining adequate uteroplacental perfusion in pregnancies with IUGR.

Another approach (Nakatsuka *et al.*, 1999) examined the effect of another NO donor, Isosorbide dinitrate (ISDN), on preeclamptic women with oligohydramnios, IUGR, and elevated resistance of blood flow in the uterine arteries. The resultant improved pulsality index (PI) of the uterine arteries and increase in amniotic fluid suggested that long-term transdermal ISDN is an effective therapy, at least in a portion of pre-eclamptic women, to avoid maternal hypertension, fetal distress, oligohydramnios, and IUGR, and consequentially to prolong the gestational period. Sieroszewski *et al.*, 2004 also conducted a NO donor study for growth restriction therapy in which the efficacy of L-arginine treatment was evaluated. There was accelerated growth in the L-arginine treated group as indicated by the ultrasound measurements of estimated fetal weight and birth weight of newborns.

2.3. Antioxidants, pregnancy, and fetal growth

2.3.1. Antioxidant Defences

Both enzymatic and non-enzymatic antioxidant systems function to protect against free radical damage, each with their own mode of action (Halliwell *et al.*, 1992, Pierce *et al.*, 2004). The enzymatic system includes SOD, which catalyzes the conversion of superoxide (O_2^-) to hydrogen peroxide (H_2O_2) and H_2O; catalase, which then converts H_2O_2 to H_2O and O_2; and GSH-Px, which reduces H_2O_2 to H_2O. The nonenzymatic antioxidants include the lipid-soluble vitamins, vitamin E and vitamin A or provitamin A (beta-carotene), and the water-soluble vitamin C and GSH (Agarwal *et al.*, 2005).

There are four different routes by which these antioxidants exert their influence (Halliwell *et al.*, 1992). One method of antioxidant action is functioning as a lipid-based free radical chain-breaking molecule leading to inhibition of lipid peroxidation and formation of oxidized LDL (Wang and Walsh, 1996). Vitamin E, mainly α-tocopherol, scavenges peroxyl radicals in the biological lipid phases such as membranes or LDL (Llurba *et al.*, 2004). Secondly, antioxidants such as glutathione may reduce the concentration of ROS. Thirdly, antioxidants may scavenge initiating radicals such as SOD, which acts in the aqueous phase to trap superoxide free radicals. Finally, a group of compounds serve an antioxidant function by sequestration of transition metals that are well-established pro-oxidants. By this method, transferrin, lactoferrin, and ferritin

function to prevent iron induced oxidant stress and ceruloplasmin and albumin as copper sequestrants (Halliwell *et al.*, 1992).

Few studies have studied the presence of antioxidants in amniotic fluid in elucidating the role it plays to counteract ROS. Jauniaux *et al.* (2004) investigated the antioxidant pathways inside the first trimester gestational sac, including 18 samples of amniotic fluid at 5–12 wk of gestation. GSH, glutathione disulfide (GSSG), and γ-tocopherol were not detectable in amniotic fluid. The mean levels of α-tocopherol, ascorbic acid, DHA, and uric acid in μmol/liter were 0.09 (SEM = 0.02), 10.0 (SEM =3.5), 1.7 (SEM =0.6), and 66.3 (SEM =7.4) respectively. There were no significant relationships between gestational age and maternal serum or amniotic fluid antioxidant values.

2.3.2. Evidence of compromised antioxidant capacity

2.3.2.1. Antioxidants in complicated pregnancies

Under normal conditions, the elevation of lipid peroxidation products is counteracted by an equal increase in antioxidant capacity and therefore has no detrimental effects. However, if the oxidative stress overwhelms the anti-oxidant defenses, damage to the membranes and other biomolecules results, leading to the underlying dysfunction of the vascular endothelium (Davidge, 1998). During normal pregnancy, SOD and catalase activity in the placenta increase with gestation, while that of GSH-P_x and α-tocopherol concentrations don't change significantly (Takehara *et al.*, 1990).

A deficiency of antioxidants may increase amounts of lipid peroxides in the maternal circulation since antioxidants limit their generation or inactivate them once they are formed. An antioxidant deficiency may explain the pathophysiology of preeclampsia. Decreased levels of vitamin E (Wang *et al.*, 1991b) and decreased antioxidant activity (Davidge *et al.*, 1992) are associated with increased levels of lipid peroxides in the maternal plasma of preeclamptic pregnancies. Some antioxidant systems have been shown to be reduced in red blood cells of preeclamptic pregnancies (Wisdom *et al.*, 1991).

Antioxidant activity or the amounts of antioxidants are also decreased in placental tissue obtained from women with preeclampsia. GSH-Px, which converts lipid peroxides to less reactive alcohols, may be inadequate in the placenta of preeclamptic women

(Hubel, 1999). Walsh and Wang (1993b) reported that placental GSH-Px activity is lower than normal in preeclamptic pregnancies and that inhibition of GSH-Px activity in normal placentas results in increased production rates of both lipid peroxides and thromboxane. In another study, the same authors (1996) found that the activities of CuZn-SOD and GSH-Px were significantly lower, whereas the activity of catalase was significantly higher, in preeclamptic pregnancies compared to normal placentas.

Plasma levels of reduced ascorbic acid, the antioxidant form, were significantly decreased in patients with mild and severe preeclampsia while plasma α-tocopherol and beta-carotene were significantly decreased only in severe preeclampsia (Mikhail *et al.,* 1994). The authors suggested that the water-soluble antioxidant nutrients, namely reduced ascorbic acid, may be consumed before the lipid-soluble antioxidants. Maternal plasma levels of other antioxidants including reduced ascorbic acid, beta-carotene, glutathione, thiol, and SOD all have been reported to be decreased in preeclampsia (Wang and Walsh, 1996). Other studies reported increased concentrations of the antioxidants GSH-Px (Uotila *et al.,* 1993) and uric acid (Bowen *et al.,* 2001) in maternal blood in preeclampsia or eclampsia, perhaps representing a compensatory response to the increased peroxide load.

Low concentrations of antioxidants have lead some researchers to hypothesize that early supplementation with antioxidants may be beneficial in decreasing oxidative stress and thereby preventing the progression of preeclampsia. The antioxidant nutrient levels can be more easily influenced by dietary or pharmacologic supplementation than the antioxidant enzymes, which are synthesized in the body (Mikhail *et al.,* 1994). In a randomized controlled trial, Chappell *et al.* (1999) concluded that supplementation at 16-22 weeks for the remaining second half of pregnancy with vitamins C and E in women at an increased risk of preeclampsia had a beneficial effect on markers of endothelial and placental function and resulted in a reduction in the proportion of women with preeclampsia. The improvement in clinical outcome suggests that ROS are involved in the pathophysiology of the disease. The mechanisms by which these antioxidants work is through inhibiting ROS. Pressman *et al.* (2003) showed that maternal plasma vitamin C and E concentrations can be increased by oral supplementation and are correlated with

vitamin C concentrations in amniotic fluid and vitamin E in the chorioamnion respectively.

On the other hand, other studies have failed to find any beneficial effects of vitamin supplementation on established severe early-onset preeclampsia (Beazley *et al.*, 2005). Stratta *et al.* (1994) chose to use 100-300 mg/day vitamin E in a non-randomized trial, while Gulmezoglu *et al.* (1997) used 1000 mg/day vitamin C, 800 IU/day vitamin E, and 200 mg/day allopurinol in a randomized controlled trial. Gulmezoglu *et al.* (1997) did however report a trend towards later delivery in the treatment group.

Decreased amounts of enzymatic and non-enzymatic antioxidants have been found in type 2 diabetics (West, 2000). An impairment of glutathione metabolism was seen in patients with diabetes (Murakami *et al.*, 1989) as well as lowered total radical-trapping antioxidant capacity in plasma of type 1 diabetics. Nourooz-Zadeh *et al.* (1997) reported significantly lowered vitamin E in plasma from Type 2 patients compared to normal controls. Vitamin E levels were also found to be lower in diabetic rats (Jain *et al.*, 1991). Kinalski *et al.* (2001) has reported decreased SOD activity in GDM placenta compared to normal controls while Toniguchi (1992) demonstrated a decreased activity of erythrocyte Cu-Zn SOD in diabetic states. Djordjevic *et al.,* (2004) observed that the SOD activity significantly decreased in the erythrocytes of mothers with Type 1 diabetes compared with healthy pregnant women. Catalase activity was significantly increased only in 3rd trimester diabetic pregnant women. Similarly, Carone *et al.* (1993) observed lower SOD activity in IDDM at each trimester and a slight increase of catalase and SOD activity during late diabetic pregnancy. On the other hand, Coughlan *et al.* (2004) demonstrated a significantly higher SOD activity in placenta obtained from women with GDM, perhaps reflecting a compensatory protective response to the existing oxidative stress present, while there was no significant difference in the activity of GSH-Px.

Pregnancies complicated by type 1 diabetes mellitus are also associated with an increased risk of fetal malformations. It has also been shown that an increased generation of ROS may play a primary role in the teratogenic process and that the addition of the scavenging enzyme SOD to the culture medium prevented glucose-induced embryopathy (Eriksson and Borg, 1991). An oversupply of nutrients to the embryo could result in excess ROS activity which could lead to enhanced lipid peroxidation. In his commentary,

Persson (2001) suggests that future research may be directed at designing clinical trials with supplementation of vitamins E and C in diabetic pregnancy.

2.3.2.2. Antioxidants and fetal outcomes

Complications of newborns are often attributed to the exposure to free radical injury (Sullivan, 1988) but oxidative stress exposes premature infants with a poorly developed antioxidant system to more oxidative damage. Both antioxidant enzymes and levels of scavengers are reportedly lower in premature newborns (Smith *et al.,* 1993, Sullivan and Newton, 1988). Reports of abnormal antioxidant defenses in fetuses have also been reported (Myatt *et al.,* 1997a, Walsh, 1998). Total antioxidant activity in serum was decreased in mothers with IUGR (Karowicz-Bilinska, 2003c). Burlingame *et al.* (2003) found antioxidant capacity to be positively correlated with gestational age and estimated fetal weights or birth weights.

CHAPTER 3: STATEMENT OF PURPOSE

Rationale

A comprehensive review of the literature reveals that different markers of oxidative (Iioka, 1994, Ishihara *et al.,* 2004) and nitrative stress (Seligman *et al.,* 1994, Myatt *et al.,* 2000) have been measured in various mediums during pregnancy including sera/plasma and amniotic fluid; however the sensitivity and specificity of the various biomarkers are not known. While some research focused on investigating the levels of oxidative stress biomarkers (Wang *et al.,* 1992, Barden *et al.,* 1996), other studies looked at antioxidant capacity (Mikhail *et al.,* 1994, Wang and Walsh, 1996) with some establishing an inverse relationship between the two opposing forces (Uotila *et al.,* 1993, Gratacos *et al.,* 1998, Mutlu-Turkoglu *et al.*, 1998). Research on antioxidant systems in pregnancy have also varied in their approach, with some measuring total antioxidant capacity (Davidge *et al.,* 1992, Toescu *et al.,* 2002) and others measuring individual enzymes such as SOD (Uotila *et al.,* 1993, Mutlu-Turkoglu *et al.*, 1998).

Of the three biomarkers proposed, only NO has been measured in amniotic fluid during second trimester (Tranquilli *et al.,* 2003) and third trimester (Di Iorio *et al.,* 1997) pregnancy. However, despite the role NO has been shown to play in both normal and abnormal pregnancy (Soorana *et al.,* 1995, Morris *et al.,* 1996, Weiner and Thompson, 1997, Sladek *et al.,* 1997), studies on the effect of a NO imbalance on the feto-placental unit have been conflicting. Its role in fetal growth and/or nitrative stress, especially in amniotic fluid, remains to be elucidated. Most studies in literature have investigated lipid peroxidation products and antioxidants as biomarkers of oxidative stress through measurements in serum/plasma; far fewer have been measured in amniotic fluid while none have investigated concentrations of TBARS and FRAP during second trimester (Mihailovic *et al.,* 2000, Burlingame *et al.,* 2003).

Main hypothesis and specific objectives

In elucidating the role of oxidative stress in amniotic fluid on human fetal growth and development, we can hypothesize that amniotic fluid biomarkers of oxidative stress captured in early gestation may explain and hence be used to predict various pregnancy outcomes. These outcome measurements include pathological pregnancy conditions such

as gestational diabetes and hypertensive disorders of pregnancy, as well as birth weight and gestational age. NO, through its influence on both pro-oxidant and antioxidant properties of the intrauterine environment, may to be an important contributor of fetal growth and/or nitrative stress. Similar to nitrative stress, the presence of lipid peroxidation products in amniotic fluid may be associated with negative pregnancy outcomes. We also propose that the antioxidant capacity of amniotic fluid will be associated with growth outcomes. Together, the investigation of these three indicators captured in the underlying oxidative status of amniotic fluid in early gestation may help understand the interrelationship of oxidative stress parameters in amniotic fluid and their influence on intrauterine fetal development.

The main objectives of this study were to: 1) assess fetal exposure to oxidative and nitrative stress in a large sample population of high risk pregnant women by measuring amniotic fluid concentrations of nitric oxide (NO), thiobarbituric acid – reactive substances (TBARS), and ferric reducing antioxidant power (FRAP) and 2) establish whether these concentrations were associated with infant birth weight, gestational age (GA), or oxidative stress-related conditions arising during pregnancy including gestational diabetes (GDM), pregnancy-induced hypertension (PIH), smoking and obesity.

CHAPTER 4: MATERIALS AND METHODS

Experimental Design and Overview

This prospective study investigated a sample population of pregnant women in Montreal, Quebec who were undergoing amniocentesis for genetic testing at St. Mary's Hospital Centre in Montreal, Quebec between the years 2000 and 2004. Amniotic fluid was obtained from mothers undergoing testing for advanced maternal age, growth assessment, and previous abnormalities among other reasons. Recruitment is ongoing since this is part of a larger study for the analysis of biomarkers in amniotic fluid; hence, the samples collected were a convenient biobank for the purpose of this study.

Ethics Approval

Ethics approval was granted from the McGill Medical Ethics Committee (Appendix A). Approval was also obtained for the hospital recruitment site and the Montreal Children's Hospital where genetic testing took place (Appendix B).

Study subjects

During recruitment, approximately 1000 women were approached and informed about the study. Written consent was obtained from the women who agreed to participate in the study and provide us with their remaining amniotic fluid. By signing the consent form, permission was given to retrieve the normally discarded portion of the fluid from Montreal Children's Hospital. Consent Forms were filed together and kept in locked file cabinets at Macdonald campus, McGill University (Appendix C).

The inclusion criteria consisted of women during second trimester pregnancy between 12 and 20 weeks gestation with singleton pregnancies. Exclusion criteria included singleton pregnancies and no genetic abnormalities since these factors may affect the results of the study. Further exclusion for long storage time was added when it was found to influence parameters, as will be discussed later on. Participants also answered a questionnaire to gather information about maternal characteristics (Appendix D). These included reported prepregnancy weight, height, age, ethnic background, smoking behavior, parity, amniocentesis week, multivitamin and/or medication use, alcohol use, coffee/tea intake, and paternal ethnic background, some of which were

further verified through medical chart review. The smoking status was assessed as three categories: current smoker and amount, quit smoking during pregnancy, or never smoked. Furthermore, the ethnic background was divided into North American, South American, European, African, Middle Eastern, Asian and other. BMI was categorized as low BMI (<18.5 kg/m^2), normal BMI (18.5-24.9 kg/m^2), overweight (25-29.9 kg/m^2), and obese (≥30 kg/m^2) (Health Canada, 2003). Infant characteristics such as infant gender, birth weight, gestational age, APGAR scores and delivery method were obtained from medical charts.

Collection of amniotic fluid samples

After completion of genetic testing, about 15 ml of sample fluid which would normally be discarded was obtained from each subject. The samples were transported to the lab where they were stored at -80°C. Amniotic fluid were matched to the appropriate consent forms and labeled with codes. Each subject was assigned a code, allowing blinding to take place. A database of 1000 amniotic fluid samples were analyzed and pair-matched with subsequent infant birth weight and/or gestational age.

Confidentiality was maintained by coding the samples. The list linking each code with the subject and data was stored in the file cabinets. Only research staff had access to the files and names were not revealed to maintain confidentiality and in line with the ethical considerations of the project.

Measured variables

There were several predictor and outcome variables. The biochemical parameters NO, TBARS and FRAP were the predictors while the outcomes parameters included both maternal and birth outcomes. Birth weight was classified in two ways: (1) gram weights in which <2500g is defined as LBW, 2500-4000g as normal birth weight, and > 4000g as macrosomia, (2) growth percentiles in which infants < 10^{th} percentile are considered SGA, those between 10% and 90% as AGA, and those > 90^{th} percentile are considered LGA. A third birth outcome variable is gestational age, was categorized into three groups: preterm (<38 wks), term (38-40.9 wks), and post-term groups (≥41 wks). Gestational age was estimated based on the mother's last menstrual period or validation by an ultrasound

examination. The large sample size allowed us to mimic the Canadian population at large and predict the size of sub-categories based on the larger population.

Ultrasound and amniocentesis measurements

Amniocentesis is a diagnostic procedure in which a small sample of amniotic fluid is drawn out of the uterus through a needle inserted in the abdomen, which is then analyzed to detect any genetic abnormalities in the fetus (Sohaey, 1998). This test is specifically common in older mothers and has instigated a new field of research regarding the study of nutritional components present in amniotic fluid as biochemical markers of fetal growth and development.

Biochemical Analysis

The development of various assays in clinical chemistry gives the ability of identifying end products of disease and markers for specific diagnosis. Such analytical procedures play an important role in providing precursors for pathological processes, allowing for the identification of the insult at an earlier stage in development. The following three methods were conducted in our laboratory.

Analysis of NO with Nitrate/Nitrite Colorometric Assay

Different methodology has been used to identify NO in physiological media or in different biological models. Since the biological half-life of NO is short, it readily undergoes a series of reactions with several molecules present in biological fluids (oxygen, superoxide, sulphydryl groups and heme), making the compound challenging for the clinical laboratory (Viinikka, 1996). The final products of NO *in vivo* are nitrite (NO_2^-) and nitrate (NO_3^-). The relative proportion of NO_2^- and NO_3^- is variable and cannot be predicted with certainty so that the best index of total NO production is the sum of both nitrite and nitrate. The Nitrate/Nitrite Colorimetric Assay Kit (Cayman Chemical, Ann Arbor, MI, USA, 2003) provides an accurate and convenient method for measurement of total nitrate/nitrite concentration and has been consistently used for NO measurement in different biological mediums including plasma and amniotic fluid (Marinoni *et al.*, 2000, Tranquilli *et al.*, 2003). Other methods used for measuring

nitrite/nitrate include HPLC, GC/MS, chemiluminescence, enzymatic assay with nitrate reductase and electron paramagnetic resonance.

The assay utilized consists of a two-step process. The first step is the conversion of nitrate to nitrite with the use of nitrate reductase enzyme. The second step is the addition of the Griess Reagents which convert nitrite into a deep purple azo compound. Photometric measurement of the absorbance due to this azo chromophore determines nitrite concentration. The contents of the kit include the nitrate/nitrite assay buffer, nitrate reductase enzyme and cofactor, nitrate and nitrite standard, Griess reagents 1 and 2, three 96-well plates and three plate cover sheets. The kit included sufficient reagents for the assay of total nitrate and nitrite.

The frozen amniotic fluid samples collected were transported to the lab and stored in the freezer at – 80°C. The samples were then thawed on ice, vortexed, and partitioned into coded Eppendorph tubes each containing around 1.5 mL of fluid. When ready for assaying, the frozen samples were thawed, filtered through a 30 kDa molecular weight cut-off filter and centrifuged for 15 minutes. The freeze-thaw process was limited to once for NO when possible for preservation of the samples. They were assayed for nitrate and nitrite by the addition of 40 μL of filtrate to each well in the plate, which was adjusted to 80 μL with assay buffer. Ten microliters of enzyme co-factors and 10 μL of nitrate reductase were added to each sample unknown and duplicate as well as the standard wells. The plate was covered and left to react at room temperature for 3 hours, the time required for complete conversion of nitrate to nitrite.

After the required incubation time, 50 μL of sulfanilamide (Griess reagent 1) followed by 50 μL of N-(1-Naphthyl)-ethylenediamine (Griess reagent 2) were added to each of the wells, standards and unknowns. The color was allowed to develop for 10 minutes at room temperature. Reagent blanks were prepared for each sample by adding 200 μL of assay buffer instead of the Griess reagents. The absorbance was determined at 540 nm using an Automated Microplate Reader (KC4, Bio-Tek Instruments, Inc., Winooski, Vermont, 2000). For each sample, a correction was made for the absorbance of the corresponding reagent blank. Duplicates which had an intervariation of greater than 10% were repeated for more accurate results. A serially diluted standard curve was constructed to quantitate sample nitrate + nitrite concentrations with a detection range of

approximately 2.5-35 μM. The standard curves produced fell within the kit's standard curve with the conversion of nitrate to nitrite close to 100%.

Analysis of lipid peroxidation with TBARS assay

There are many currently available assays for measuring lipid peroxidation. These measurements are taken at different points on the peroxidation chain such as measuring the primary peroxidation products (lipid hydroperoxides), or various secondary products (such as MDA). When measured with Kamiya Biomedical Company's LPO assay kit (Seattle, WA), levels of lipid hydroperoxides in human amniotic fluid, were below the limit of detection of the assay which may be attributed to the lack of sensitivity of the assay. Unfortunately, most lipid peroxidation assays have sensitivity and specificity problems. There is no single standardized, reliable clinical measurement of the process of lipid peroxidation (Little and Gladen, 1999).

The sensitivity of measuring TBARS has made this assay the most widely used method for screening and monitoring lipid peroxidation. Although this reaction is very sensitive, its specificity, even with improvement of pre-analytical (sampling, preservatives) and analytical stages (fluorescence, HPLC) is still in question. MDA participates in reactions with molecules other than 2-thiobarbituric acid (TBA) and is a catabolic substrate. Only certain lipid peroxidation products generate MDA. In addition, MDA is not the only product of fatty acid formation and decomposition and is not a substance generated solely from lipid peroxidation.

Currently, the concept of TBARS has gradually replaced the initial MDA assay, which only considers one of the end-products formed during oxidative stress in its specific determination of MDA. Future research should explore precise analytical determination of different molecules triggered by free radicals in the assessment of oxidative stress. The TBARS assay should be considered as a global test, allowing a global approach of lipid peroxidation and serves as a useful parameter for general cellular oxidative damages (Lefevre, 1998).

Biological specimens contain a mixture of TBARS, including lipid hydroperoxides and aldehydes. The presence of anti-oxidants will influence the levels of TBARS which return to normal levels over time. Unless assayed immediately, samples

should be frozen at – 80C to prevent loss of MDA and 4-hydroxyalkenals (HAE), other decomposition products of lipid peroxidation, and prevent new sample oxidation. The lipid material is simply heated with TBA at a low pH and high temperature, and the formation of a pink chromogen is measured spectrophotometrically at 532 nm. The chromogen is formed by the reaction of one molecule of malondialdehyde (MDA) with two molecules of TBA. Several other aldehydes formed in peroxidizing lipid systems give different chromogens with TBA. The original 1:2 MDA:TBA adduct can be separated by HPLC from these other chromogens before measurement. Butylated hydroxytoluene was added to prevent any initiation of membrane lipid peroxidation during the assay. Its addition to the standard MDA does not affect its color development with the TBA. The MDA standard is used to create a standard curve against which unknown samples can be plotted (Janero, 1998).

Analysis of antioxidant capacity with FRAP assay

The Ferric Reducing/Antioxidant Power Assay (FRAP) is a novel method for assessing "total antioxidant power" (Benzie and Strain, 1996). The FRAP assay is inexpensive, reagents are simple to prepare, results are highly reproducible, and the procedure is straightforward and speedy. Reduction of ferric to ferrous ion at low pH causes a blue-colored ferrous-tripyridyltriazine complex to form. FRAP values are obtained by comparing the absorbance change at 593 nm in test reaction mixtures with those containing ferrous ions in known concentration. Absorbance changes are linear over a wide concentration range with antioxidant mixtures and with solutions containing one antioxidant in purified form. There is no apparent interaction between antioxidants (Benzie and Strain, 1999).

CHAPTER 5

MANUSCRIPT

Oxidative and Nitrative Stress in Second Trimester Amniotic Fluid are Associated with Pregnancy Outcomes

Dima El-Halabi, Kebba Sabally, Stan Kubow, Kristine G. Koski

School of Dietetics and Human Nutrition, McGill University (Macdonald Campus), Montreal, Canada H9X-3V9

Corresponding Author: Dr. Kristine G Koski, Director and Associate Professor, School of Dietetics and Human Nutrition, Macdonald Campus, McGill University, 21,111 Lakeshore, Ste. Anne de Bellevue, Quebec, Canada H9X-3V9, Ph: (514)398-7845, Fax: (514) 398-7739
Email: Kris.Koski@mcgill.ca

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ABSTRACT

The main objective of this study was to assess whether fetal exposure to oxidative and nitrative stress in amniotic fluid as measured by nitric oxide (NO), thiobarbituric acid–reactive substances (TBARS), and ferric reducing antioxidant power (FRAP) was associated with infant birth weight and gestational age (GA) in normal human pregnancy. A biobank of frozen second trimester amniotic fluid samples from 198 mothers undergoing amniocentesis for genetic testing in Montreal, QC, Canada was analyzed. Maternal and neonatal characteristics were collected from medical charts and questionnaires. ANOVAs and multivariate regression analyses, controlling for maternal height, prepregnancy weight, smoking behavior, infant gender, parity, delivery method, and sample storage length showed that NO differed among pre-term, term, and post-term infants and was a positive predictor of gestational age. TBARS concentrations were highly correlated with length of sample storage but were not associated with pregnancy outcomes; whereas amniotic fluid FRAP positively predicted gender-corrected birth-weight-for-gestational-age. Our study suggests that amniotic fluid measures of oxidative and nitrative stress early in pregnancy are associated with birth weight and gestational age.

KEY WORDS: amniotic fluid, oxidative stress, nitric oxide, lipid peroxidation, antioxidant, gestational age, birth weight

INTRODUCTION

Oxidative stress occurs when there is an excessive production of reactive oxygen species (ROS) or a deficiency of antioxidants, resulting in damage to proteins, lipids, and DNA (Hubel *et al.*, 1989, Halliwell, 1994). A state of oxidative stress can arise through normal physiological processes, including an increase in lipid peroxidation and antioxidation reactions across gestation, as well as disease states. There is evidence that lipid peroxides, established markers of oxidative stress, increase in normal pregnancy compared to the nonpregnant state but that this increase is offset by increased concentrations of antioxidants such as vitamin E (Wang *et al.*, 1991) and increased placental activities of superoxide dismutase (SOD) and catalase as gestation progresses (Sekiba and Yoshioka, 1979, Takehara *et al.*, 1990).

Oxidative and nitrative stress, caused by the reaction of ROS and nitric oxide (NO), have been implicated in multiple complications of pregnancy, including diabetes and preeclampsia (Walsh, 1994, Hubel, 1999, Myatt *et al.*, 2004), which have also been related to adverse fetal growth. Studies that support the role of oxidative stress in preeclampsia include evidence of an increase in circulating plasma lipid peroxides (Hubel *et al.*, 1996, Gratacos *et al.*, 1998, Walsh *et al.*, 2000) and a decrease in plasma antioxidants in preeclamptic patients (Davidge *et al.*, 1992, Uotila *et al.*, 1993, Mikhail *et al.*, 1994, Mutlu-Turkoglu *et al.*, 1998). Decreased plasma antioxidant concentrations has formed the basis for preventative trials using antioxidant supplementation in preeclampsia, which have shown either a beneficial effect on clinical outcome (Chappell *et al.*, 1999) or no significant clinical improvements (Gulmezoglu *et al.*, 1997, Beazley *et al.*, 2005).

Suboptimal fetal growth, resulting in poor pregnancy outcomes such as low birth weight and prematurity, has also been associated with oxidative stress (Myatt *et al.*, 2004). Various parameters reflecting oxidative damage including malondialdehyde (MDA), lipid hydroperoxides and conjugated dienes were significantly increased in placenta, maternal serum, or cord blood of intrauterine fetal growth retarded (IUGR) pregnancies compared to normal pregnancies (Karowicz-Bilinska *et al.*, 2002, 2004, Takagi *et al.*, 2004, Kamath *et al.*, 2006). Intrauterine malnutrition was associated with significant oxidative stress in cord blood in small-for-gestational age (SGA) neonates born at term to malnourished mothers (Gupta *et al.*, 2004). Moreover, placental total F_2-isoprostanes (F2-IP) were

significantly higher in preterm deliveries than in term deliveries (Comporti *et al.*, 2004). Analysis of amniotic fluid from IUGR pregnancies showed higher MDA concentrations at parturition (Bazowska *et al.,* 1994) and increased F2-IP concentrations in the second trimester (Longini *et al.,* 2004).

The role of NO in pregnancy goes beyond its influence on nitrative-stress induced pathological conditions of pregnancy to acting as a regulatory molecule in normal maternal adaptations to pregnancy and fetal growth. NO is synthesized from L-arginine by a family of enzymes called nitric oxide synthases (NOS). It has been shown to maintain the uteroplacental circulation in a vasodilated state to ensure adequate delivery of oxygen and nutrients to the growing fetus and remove metabolic waste (Rosselli *et al.*, 1998, Buhimschi *et al.*, 1998). As NO contributes to smooth muscle relaxation, NO activity in the uterus may contribute to the maintenance of uterine quiescence during pregnancy and its withdrawal prior to term may prompt parturition (Buhimschi *et al.*, 1998). Increased expression of myometrial inducible NOS (iNOS) in rats (Riemer *et al.*, 1997) and humans (Bansal *et al.*, 1997) has been demonstrated during pregnancy that declines towards term. Due to its vasodilatory effects and implication in angiogenesis, an impairment in NO may reduce fetoplacental circulation in pregnancies with higher vascular resistance such as preeclampsia and IUGR (Sladek *et al.,* 1997, Norris *et al.,* 1999). However, studies are equivocal as elevated (Smarason *et al.,* 1997, Ranta *et al.*, 1999), unaltered (Wang *et al.,* 1994, Davidge *et al.,* 1996) or diminished plasma NO concentrations (Seligman *et al.,* 1994) have been noted in preeclampsia. In terms of amniotic fluid, Tranquilli *et al.* (2003) and DiIorio *et al.* (1997) showed increased amniotic fluid NO concentrations in the third trimester during IUGR, while lower concentrations were seen in second trimester IUGR (Tranquilli *et al.,* 2003) NO may contribute to nitrative stress in the presence of varying concentrations of ROS and antioxidants (Bloodsworth *et al.,* 2000). The interaction of NO and superoxide produces peroxynitrite, a powerful prooxidant with diverse deleterious effects, including nitration of tyrosine residues on proteins. Kossenjans *et al.* (2000) observed increased expression of nitrotyrosine residues, an indirect indication of excessive ROS generation, in the fetal vasculature and villous stroma of preeclamptic and diabetic placentas and pregnancies with altered placental function (Myatt *et al.*, 2004). Whether it mediates maternal

vasodilation in normal pregnancy or maintains uterine quiescence across gestation, NO's potential effects on length of gestation and birth weight within the amniotic fluid medium require further investigation.

The assessment of amniotic fluid constituents as prognostic indicators of adverse pregnancy outcomes is under active investigation. Despite increasing evidence suggesting the importance of oxidative and nitrative stress in pathogenic outcomes during pregnancy, there are surprisingly few studies that have examined these relationships using amniotic fluid biomarkers, particularly in normal pregnancy. Our study intent was to assess whether earlier conditions of oxidative and nitrative stress would impact subsequent birth weight and gestational age. The assessment of oxidative stress biomarkers and antioxidant defenses during second trimester may provide support for the utilization of such markers as etiologic predictors of adverse pregnancy outcomes. Hence, the main study objective to assess to establish whether fetal exposure to oxidative and nitrative stress as measured by amniotic fluid concentrations of nitric oxide (NO), thiobarbituric acid–reactive substances (TBARS), and ferric reducing antioxidant power (FRAP) was associated with infant birth weight and gestational age in normal pregnancies.

MATERIALS AND METHODS

Design, recruitment and consent. From 2000 to 2003, pregnant women undergoing routine amniocentesis at St Mary's Hospital Center (Montreal, Canada) were approached to participate in the study. Ethics approval was obtained from the Institutional Review Boards of McGill, Montreal Children's Hospital, and St Mary's Hospital Center where amniocentesis was conducted. The subjects were asked to sign consent forms which allowed researchers to obtain amniotic fluid once genetic testing was completed. Samples were stored at -80°C until analysis. Exclusion criteria included genetic abnormalities, multiple pregnancies, and sample storage length greater than two years. Maternal and infant characteristics were collected from chart review and subject questionnaires, including maternal age, prepregnancy weight, height, smoking behavior, amniocentesis week, parity, ethnicity, delivery method, maternal diseases, infant gender, gestational age, birth weight, and APGAR scores. Birth-weight-corrected-for-gestational-

age categories were subsequently calculated from infant gender, gestational age, and birth weight (Kramer, 1987).

Biochemical analysis

Nitric oxide (NO)

The final products of NO *in vivo* are nitrite (NO_2^-) and nitrate (NO_3^-). The relative proportion of nitrite and nitrate is variable and cannot be predicted with certainty so that the best index of total NO production is the sum of both. The Nitrate/Nitrite Colorimetric Assay Kit (Cayman Chemical, Ann Arbor, MI, USA) was used to measure total nitrate/nitrite concentration as has been consistently used for NO measurement in different biological fluids including plasma (Marinoni *et al.*, 2000) and amniotic fluid (Marinoni *et al.*, 2000, Tranquilli *et al.*, 2003).

Samples were thawed on ice, filtered through a 30 kDa molecular weight cut-off filter and centrifuged. Total nitrite and nitrate was assayed using 40 μL of sample and addition of 10 μL of enzyme co-factors and 10 μL of nitrate reductase to sample duplicates and standards in 96-well Microtest III plates. The plate was left to react at room temperature for three hours for complete conversion of nitrate to nitrite. After the required incubation time, 50 μL of sulfanilamide (Griess reagent 1) followed by 50 μL of N-(1-Naphthyl)-ethylenediamine (Griess reagent 2) were added and the color was allowed to develop for 10 min at room temperature. Reagent blanks were prepared for each sample by adding 200 μL of assay buffer instead of the Griess reagents. The absorbance was read at 540 nm using an Automated Microplate Reader (KC4, Bio-Tek Instruments, Inc., Winooski, Vermont, 2000). A serially diluted standard curve was constructed to quantitate sample nitrate and nitrite concentrations with a detection range of approximately 2.5-35 μM. The conversion of nitrate to nitrite was nearly 100%.

Thiobarbituric acid-reactive substances (TBARS)

The TBARS method is considered a global assay for measuring lipid peroxidation and serves as a useful parameter for general oxidative damages (Lefevre, 1998). TBARS concentrations were measured using methods adapted from Asakawa and Matsushita (1979) and Wong *et al.* (1987) and modified by Liu *et al.* (2007). Samples were heated

with 2-thiobarbituric acid (TBA) at a low temperature and high pH resulting in the formation of a 1:2 MDA:TBA colored complex, which was quantified spectrophotometrically at 532 nm in a microplate reader (Series 750, Cambridge Technology, Inc., Cambridge, MA). A standard curve was created with which to quantify the TBARS concentrations in the samples. TMOP (1,1,3,3-tetramethoxypropane) in the stock solution is converted to MDA upon heating and binds to TBA forming TBARS. The intensity of the color increases with increasing concentrations of TMOP; thereby, forming the standard curve.

Ferric reducing/antioxidant power assay (FRAP)

We measured total antioxidant power using FRAP according to Benzie and Strain (1999). The FRAP assay assesses "total antioxidant power" using antioxidants as reductants in a redox-linked colorimetric method. Hence, the Fe3+-TPTZ (ferric tripyridyltriazine) complex is reduced at a low pH to the blue ferrous form, Fe2+. The procedure involves mixing 300 μL of freshly prepared FRAP reagent containing 300 m*M* acetate buffer, pH 3.6, 10 m*M* TPTZ in 40 m*M* HCl, and 20 m*M* $FeCl_3 \cdot 6H_2O$ in the ratio of 10:1:1 with 30 μL of water and 10 μL of sample. The absorbance at 593 nm was taken at 6 and 12 min after incubation at room temperature using an Automated Microplate Reader (KC4, Bio-Tek Instruments, Inc., Winooski, Vermont, 2000). The change of absorbance was calculated and related to the change of absorbance of the standard solution. Aqueous ascorbic acid solutions in the concentration range of 100 to 1000 μ*M* were used for calibration of the FRAP assay. The change of absorbance is linearly proportional to the concentration of antioxidant. FRAP values derived from duplicate analysis were expressed in μ*M* units.

Statistical Analysis. All data were analyzed using SAS (Version 9.1, SAS Institute). Data were reported as means ± SEM unless otherwise indicated. All non-normally distributed data (maternal age, BMI, parity, gestational age, amniocentesis week, NO, and TBARS) were $\log_{10}$ transformed. Pearson and Spearman correlations were performed between the oxidative stress parameters and various pregnancy outcome variables as well as storage length. ANOVA and ANCOVA were performed across population subgroups based on

clinical classifications of gender-corrected birth-weight-for-gestational-age categories, consisting of small-for-gestational-age (SGA; $<10^{th}$ percentile), appropriate-for-gestational-age (AGA; 10-90%), and large-for-gestational-age (LGA; $\geq$90%) (Kramer *et al.,* 2001). Similarly, subgroups for gestational age were created consisting of preterm (<38 wks), term (38-40.9 wks), and post-term groups ($\geq$41 wks). We chose less than 38 weeks for the prematurity subgroup rather than less than 37 weeks since recent data has suggested the following two points: 1) the last menstrual period estimation method often underestimates prematurity due to delayed ovulation (Kramer *et al.,* 1992); and 2) according to the WHO, gestational age at birth should be estimated in completed weeks and physicians will often round up births between 37 and 38 weeks to 38 weeks (Wen *et al.,* 2003). Differences across delivery methods, amniocentesis week, and varying APGAR scores were also tested. Low APGAR scores were defined as below 7 and normal APGAR scores defined as 7-10 (Casey *et al.,* 2001). Post-hoc testing was performed using Scheffe's test ($P \leq 0.05$). Covariates for analysis included pre-established predictors of birth weight: maternal height, pre-pregnancy weight, ethnicity, parity, gender and gestational age, smoking status (Kramer, 1987) and variables which were found to differ across birth outcome categories. Multiple linear regressions were performed with each biochemical parameter entered separately. Significance was set at $P \leq 0.05$.

RESULTS

Population Characteristics

The study population consisted of 198 mother-infant pairs who met the exclusion criteria. The majority of the women were non-smokers (88.5%) and 61.4% had a normal prepregnancy BMI (defined as a BMI between 18.5-24.9 kg/m^2). The mean birth weight was 3473 $\pm$ 38 g. Women with complications of pregnancy included GDM (10%) and PIH (3%). When subdivided by gender-corrected birth-weight-for-gestational age classifications, 5% were SGA, 82% were AGA, and 13% were LGA. Using gestational age categories, 11% were preterm infants, 80% were born at term, and 9% were post-term infants.

The different maternal and fetal variables were compared across the gender-corrected birth weight percentiles (Table 1a) and gestational age categories (Tables 1b). Variables that differed across birth-weight-for-gestational-ages included maternal pre-pregnancy weight and height. The variables that differed across gestational age categories. i.e., the pre-term, term and post-term groups, included birth weight and amniocentesis week. As a result, these latter measures were included as covariates in our statistical analysis in addition to pre-established predictors of birth weight (Kramer, 1987).

Biochemical Parameters in Amniotic Fluid

The concentrations of each parameter were tested for differences that might arise due to storage length over this period (≤ 2 years). TBARS were correlated with storage length ($r = 0.335$, $P < 0.0001$), while NO and FRAP were not. Concentration differences across the week of gestation in which the amniocentesis was performed, were not significant.

All three biochemical measures of oxidative stress did not differ across the three delivery methods: Cesarean-section, induced labor, and spontaneous vaginal delivery. The oxidative stress parameters did not differ between infants with low APGAR scores and normal APGAR scores either at 1 min and 5 min post-partum (results not shown).

Nitric oxide (NO)

NO was positively correlated with gestational age by Pearson's correlations ($r = 0.16$, $P = 0.028$) and Spearman's correlations ($r = 0.17$, $P = 0.014$). NO did not differ across gender-corrected birth-weight-for-gestational-age categories but differed across gestational age categories of preterm, term, and post-term even after inclusion of covariates (Table 2). Multiple linear regression showed that NO did not predict gender-corrected birth-weight-for-gestational-age (Table 3a); however, it was a positive predictor of gestational age ($P=0.0044$, $R^2=11.31$) (Table 3b).

Thiobarbituric acid–reactive substances (TBARS)

TBARS did not differ across the gender-corrected birth-weight-for-gestational-age categories with inclusion of the covariates maternal height, pre-pregnancy weight,

ethnicity, parity, delivery method, smoking status and storage time (Table 2). In contrast, TBARS differed across preterm, term and post-term groups; however, differences no longer remained significant after including the covariates of ethnicity, parity, delivery method, amniocentesis week and storage time. Multiple linear regression showed that TBARS concentrations were not a significant predictor of birth weight or gestational age (Table 3a,b).

Ferric reducing antioxidant power (FRAP)

FRAP did not differ across gender-corrected birth-weight-for-gestational age groups or gestational age categories (Table 2). On the other hand, using regression analyses, FRAP positively predicted birth weight ($P=0.0416$, $R^2=44.76$) (Table 3a), although FRAP was not a significant predictor of gestational age (Table 3b).

DISCUSSION

The disturbed balance between ROS/reactive nitrogen species (RNS) and the antioxidant defenses produces a state of oxidative stress that has been implicated in multiple complications of pregnancy in both humans and animals (Hubel *et al.,* 1989, Carone *et al.,* 1993, Barth *et al.,* 1994) but its impact on normal growth and development is relatively unexplored. Oxidative stress has been hypothesized to act as the link between adverse insults associated with preterm birth and low birth weight, including preeclampsia, diabetes, smoking, and malnutrition, and fetal programming of adult diseases (Luo *et al.,* 2006). Studies to this date have usually measured lipid peroxidation markers and antioxidants in maternal and fetal serum, while NO activity has been studied in amniotic fluid in third trimester in view of its role as a mediator of uterine function or in pregnancy pathological states. The aim of the present work was to establish whether any of the oxidative and nitrative biomarkers NO, TBARS or FRAP, measured in second trimester amniotic fluid, were associated with infant birth weight and gestational age. The major findings were that gestational age was predicted by amniotic fluid concentrations of NO at the second trimester and that infant birth weight was positively associated with FRAP concentrations in second trimester amniotic fluid.

Our data consisted of samples from 198 pregnancies, consisting of predominantly healthy women who were multiethnic (62.5% Caucasian, 11.5% Asian, and 26.0% Middle Eastern, Black, and South American) with a normal BMI (61.4%, 24.2 ± 0.35), non-smokers (88.5%), and delivered by spontaneous vaginal delivery (48.5%). The incidence of SGA at 5% was slightly lower than the Canadian average of 7.9%; however, the incidence of LGA was 13%, which were similar to the 12.0% for the Canadian population at large (Canadian Perinatal Health Report, 2003). The incidence of prematurity, gestational diabetes, and pregnancy induced hypertension was 12%, 7.0%, and 4.6%, respectively. Pre-established determinants of infant birth weight, including prepregnancy weight, height and parity (Kramer, 1987), were found to be predictors in our population, indicating that our older women (average age of 37.5) behaved similarly to the Canadian population at large.

In the present study, we have measured amniotic fluid NO concentrations much earlier (gestational ages 12-22 wks) than previously reported and have found that NO positively predicts gestational age in a large sample population in both preterm and post-term deliveries. While previous studies have reported on the role of NO in maintaining uterine and feto-placental blood flow in normal and abnormal pregnancy as well as uterine quiescence during pregnancy, the results of the present study can be interpreted as supporting a role of NO in the modulation of gestational age later in pregnancy. NO has been thought to relax the uterine myometrium by increasing guanosine 3′, 5′- cyclic monophosphate (cGMP) (Sladek *et al.,* 1997). The decrease in NOS activity may be as early as second trimester to produce an effect on the onset of labor with advancing gestation. Changes in levels of NO across gestation related to NO's role in the maintenance of uterine quiescence have been observed by Morris *et al.* (1995b) who reported a reduction of amniotic fluid NO concentrations after 37 weeks of gestation and von Mandach *et al.* (2003) who showed increased amniotic fluid levels from first trimester to third trimester with a slight decline at term. Other amniotic fluid studies found no correlation between gestational age and NO concentrations collected at parturition (Marinoni *et al.,* 2000) or third trimester in normal and IUGR pregnancies (DiIorio, 1997). Two studies that found a positive correlation between NO and gestational age included Tranquilli *et al.* (2003) who found the correlation in third

trimester amniotic fluid when IUGR was diagnosed and Egberts *et al.* (1999) who observed this relationship in pregnancies at risk of alloimmunization in amniotic fluid obtained between 23 and 37 weeks. On the other hand, Norris *et al.* (1999) found a negative correlation between NO levels in the fetoplacental circulation of preeclamptic pregnancies and gestational age but suggested that the correlation may have been due to the severity of fetal growth restriction rather than to gestational age. This study is not comparable to our study since we used birth weight data that were corrected for gender and gestational age and it occurred in a different medium. Comporti *et al.* (2004) reported that plasma isoprostane levels were inversely correlated with gestational age in newborns, term and preterm infants.

Due to its effect on vascular reactivity, the hypothesis that either decreased NO biosynthesis within the vasculature of preeclamptic and IUGR pregnancies or NO up-regulation in impaired perfusion has been previously tested. Our study found no associations between NO and birth weight possibly given its lack of expression in a normal population but rather in IUGR pregnancies which were not evaluated. Various studies have reported increased NO production in the fetoplacental unit during IUGR (Morris *et al.,* 1995a, Lyall *et al.*, 1996, DiIorio *et al.*, 1997, Myatt *et al.,* 1997) and both elevated (Wijnberger *et al.,* 2001) and reduced (Hata *et al.*, 1998) NO metabolites in SGA infants after birth. Hence, our study results support the concept that NO may play a role in normal human physiology of pregnancy, particularly in relation to the timing of parturition.

Our study found a positive relationship between amniotic fluid antioxidant capacity and infant birth weight; however no relationship was found with gestational age. Antioxidant systems have been shown to be modified during normal pregnancy to control lipid peroxidation (Gitto *et al.*, 2002). In early pregnancy, the increase in oxygen tension is associated with the increase in mRNA of the antioxidant enzymes catalase, glutathione peroxidase, and SOD within placental tissues (Myatt *et al.,* 2004). Various antioxidants such as vitamin E (Uotila *et al.,* 1991) and ceruloplasmin (Wisdom *et al.,* 1991) have been shown to increase with gestation in placenta. Neonates, particularly preterm and SGA infants, may be at a greater risk of oxidative stress due to their decreased ability to bind transition metals and low circulating levels of lipophilic antioxidants (Sullivan and

Newton, 1988, Lindeman *et al.,* 1992). Reports of abnormal antioxidant defenses in fetuses have been previously reported (Myatt *et al.,* 1997, Walsh, 1998). Oxidative stress exposes premature infants with a poorly developed antioxidant system to further oxidative damage (Sullivan and Newton, 1988, Smith *et al.,* 1993) and to free radical injury (Sullivan, 1988). Vento *et al.* (2000) measured total antioxidant activity and uric acid concentrations in the blood of 16 mechanically ventilated preterm infants and found it to be undetectable or very low in babies not requiring O_2 therapy, while highest levels of uric acid were found in babies ventilated with 100 % oxygen.

Elevated umbilical venous isoprostane levels was an important determinant of adverse neonatal outcome, suggesting that in utero oxidative stress may lead to adverse pregnancy outcomes (Weinberger *et al.,* 2006). Total antioxidant activity in serum was decreased in mothers with IUGR infants (Karowicz-Bilinska, 2003). Although some researchers showed positive correlations between antioxidant capacity of cord blood plasma and gestational age (Sullivan and Newton, 1988, Silvers *et al.*, 1998), Lindman *et al.* (1989) did not observe a difference between pre-term and full-term status when measuring antioxidant capacity by the total radical trapping capacity assay (TRAP). With respect to amniotic fluid, Burlingame *et al.* (2003) found a positive relationship between total antioxidant capacity in amniotic fluid measured in second and third trimester and advancing gestational age as well as fetal weight. No differences were observed in levels of retinol and vitamin E in amniotic fluid of pregnant women with or without preterm premature rupture of membranes (PROM); however, significantly lower levels of amniotic fluid vitamin C were seen in patients with preterm PROM (Barrett *et al.,* 1994). We observed lower expression of antioxidant capacity on the SGA groups, reflecting either an inefficient transfer of antioxidants or increased fetal utilization due to increased lipid peroxidation; hence the maintenance of an adequate antioxidant concentration may result in a more favourable fetal outcome. In view of the large variability captured by FRAP as a predictor of birth weight in the present work, our results may implicate the role in which antioxidants play in utero to promote fetal growth.

Of the several methods available for measuring total antioxidant capacity, the FRAP method was selected because it directly measures antioxidants or reductants in a sample, requires very small sample volumes, has constant stoichiometric factors and a

general selectivity for electron-donating antioxidants, and provides an absolute quantitative determination of the total amounts of antioxidants or reductants in samples (Benzie and Strain, 1999). However, a limitation to FRAP is that it predominantly measures the antioxidant capacity of water soluble antioxidants, namely uric acid, vitamin C and bilirubin in plasma, while hydrophobic components, glutathione and protein thiols contribute to a lesser extent to the total FRAP value (Cao and Prior, 1998). The total antioxidant capacity of amniotic fluid as measured by FRAP, however, is likely similar to plasma, which mainly arises from albumin, uric acid, vitamin C and bilirubin. Due to the lack of a standard assay for measuring antioxidant capacity, further assay analysis can be applied to validate our method.

Some studies suggest that various obstetric complications or the method of delivery has an impact on oxidative stress in both mother and fetus (Rao *et al.*, 2003). Lipid peroxidation reportedly is increased in erythrocytes of fetuses born after premature rupture of membranes (Rao *et al.*, 2003) and in erythrocytes of mothers with prolonged second stage of labor and delivery through lower segment cesarean section (Yin *et al.*, 1995). Moreover, Inanc *et al.* (2005) reported that plasma MDA levels were lower in the cesarean section vs. the vaginal delivery group while superoxide dismutase and catalase activities were higher in the cesarean group, suggesting that the antioxidant defenses are increased to overcome oxidative stress in newborns delivered by C-section. In our study, we did not find associations between our biochemical parameters and delivery method (results not shown). Although we used the TBARS method as a global assessment of oxidative stress present in the amniotic fluid, the measurements were strongly influenced by storage length; hence, any relationship was lost after correcting for storage. The determination of F2-isoprostanes (F2-IP), oxidation products of arachidonic acid, has been proposed as a more reliable index of oxidative stress (Abuja and Albertini, 2001). Comporti *et al.* (2004), however, reported no significant differences between the plasma F_2-IP values for term and preterm newborns with vaginal delivery and those with cesarean section, also suggesting that the delivery method may not have an impact on the increased oxidative stress. However, they did find a significant negative correlation between the plasma isoprostane levels and the APGAR scores at 5 min after birth whereas in our study, our oxidative stress parameters did not differ between infants with low

APGAR scores and normal scores, suggesting in future studies that amniotic fluid isoprostanes should be measured.

Our study revealed that biomarkers of oxidative and nitrative stress in utero are associated with pregnancy outcomes. Targeting ways to modulate NO levels in utero could allow prevention of prematurity and minimize post-term deliveries. Moreover, our positive associations between FRAP and birth weight strongly suggests that higher antioxidants in utero as early as second trimester will result in healthier, larger birth weight infants. Further research on the role of oxidative/nitrative stress biomarkers in the amniotic fluid is warranted.

TABLE 1a. Differences in maternal, infant and human amniotic fluid characteristics for the entire population (n=191-198) across gender-corrected birth-weight-for-gestational-ages: SGA (< 10%), AGA (10-90%), and LGA (>90%)[1]

Characteristic	SGA	AGA	LGA	F	P
Infant					
Birth weight, *g*	2740 ± 56[a]	3386 ± 33[b]	4308 ± 89[c]	68.79	<.0001
Gender, *%female*	55.6	47.6	44.0	0.18	NS
Gestational age, *wk*	39.8 ± 0.27	39.4 ± 0.12	39.6 ± 0.35	0.48	NS
Maternal					
Height, *m*	1.57 ± 0.019[a]	1.62 ± 0.006[a]	1.66 ± 0.010[b]	6.65	0.0016
Prepregnancy weight, *kg*	55.1 ± 4.3[a]	62.9 ± 1.0[a]	71.6 ± 2.6[b]	6.89	0.0013
BMI, kg/m^2	22.5 ± 1.6	24.0 ± 0.4	26.0 ± 1.0	2.31	NS
Ethnicity, *%*				2.28	NS
Nonsmoking, *%*	100.0	87.3	91.7	0.80	NS
Parity	0.67 ± 0.17	1.18 ± 0.09	1.24 ± 0.20	0.62	NS
Delivery method, *%*				1.41	NS
Amniocentesis week, *wk*	15.6 ± 0.46	15.7 ± 0.10	15.6 ± 0.25	0.12	NS

[1]Values are means ± SEM or % of 9 SGA, 164 AGA and 25 LGA infants. Means in a row with superscripts without a common letter differ, $P \leq 0.05$. NS = not significant ($P > 0.05$)

TABLE 1b. Differences in maternal, infant and human amniotic fluid characteristics for the entire population (n=191-198) across gestational age categories: preterm, term, and post-term[1]

Characteristic	Preterm	Term	Post-term	F	P
Infant					
Birth weight, *g*	2962 ± 139[a]	3476 ± 37[b]	4049 ± 92[c]	24.63	<.0001
Gender, *% female*	47.6	47.8	44.4	0.04	NS
Gestational age, *wk*	36.4 ± 0.39[a]	39.6 ± 0.06[b]	41.4 ± 0.07	152.38	<.0001
Maternal					
Height, *m*	1.61 ± 0.013	1.62 ± 0.006	1.63 ± 0.015	0.55	NS
Prepregnancy weight, *kg*	63.2 ± 2.9	63.6 ± 1.1	64.8 ± 3.0	0.08	NS
BMI, kg/m^2	24.5 ± 1.1	24.1 ± 0.4	24.4 ± 1.2	0.07	NS
Ethnicity, *%*				2.67	NS
Nonsmoking, *%*	94.7	86.5	100.0	1.70	NS
Parity	1.19 ± 0.20	1.20 ± 0.09	0.89 ± 0.23	1.09	NS
Delivery method, *%*				0.12	NS
Amniocentesis week, *wk*	16.3 ± 0.39[a]	15.5 ± 0.08[b]	16.3 ± 0.51[a]	5.52	0.0047

[1]Values are means ± SEM or % of 21 preterm newborns, 159 term newborns, and 18 post-term newborns. Means in a row with superscripts without a common letter differ, $P \leq 0.05$. NS = not significant ($P > 0.05$)

TABLE 2. Differences in biochemical parameters across gender-corrected birth-weight-for-gestational-ages and gestational age categories[1]

Birth Outcome	NO (μM)	TBARS(μM)	FRAP(μM)
Percentiles[2]			
SGA (<10%)	(9) 30.8 ± 5.2	(8) 3.02 ± 0.50	(7) 879.8 ± 53.5
AGA (10-90%)	(164) 32.8 ± 1.2	(120) 3.68 ± 0.13	(75) 817.1 ± 16.3
LGA (> 90%)	(25) 31.8 ± 3.1	(15) 3.75 ± 0.37	(4) 952.3 ± 70.8
Gestational age[3]			
Preterm (<38)	(21) 26.6 ± 3.6^{a}	(17) 3.02 ± 0.34^{A}	(6) 855.9 ± 59.0
Term (38-40.9)	(159) 31.9 ± 1.2^{a}	(112) 3.74 ± 0.13^{B}	(75) 822.8 ± 16.7
Post-term (≥41)	(18) 43.4 ± 3.7^{b}	(14) 3.71 ± 0.38^{AB}	(5) 880.8 ± 64.6

[1] Values are means ± SEM. Means in a column with superscripts without a common letter differ, $P \leq 0.05$. n=198 for NO, n=143 for TBARS, and n=86 for FRAP.

[2]Covariates include maternal height, pre-pregnancy weight, parity, ethnicity, delivery method, smoking status and storage time.

[3]Covariates include ethnicity, parity, delivery method, and storage time. Without storage time as a covariate, TBARS were significantly different among gestational age subcategories (shown in capital letters).

TABLE 3a. The oxidative stress parameters as independent predictors of gender-corrected birth-weight-for-gestational ages[1]

Variable	Gender-corrected birth-weight-for-gestational age					
	Model 1		Model 2		Model 3	
	β±SEM	P	β±SEM	P	β±SEM	P
Amniotic fluid						
NO, *μM* (n=184)	0.004 ± 0.12	0.9744				
TBARS, *μM* (n=132)			0.18 ± 1.75	0.9200		
FRAP, *μM* (n=72)					0.04 ± 0.02	0.0416
Maternal/Infant Variables						
Prepregnancy wt, *kg*	0.5 ± 0.2	0.0183	0.4 ± 0.2	0.0154	0.6 ± 0.2	0.0014
Height, *m*	65.7 ± 28.4	0.0219	88.7 ± 32.8	0.0078	84.5 ± 33.9	0.0152
Smoking behavior[2]	11.6 ± 6.4	0.0733	7.6 ± 7.7	0.3260	11.5 ± 7.7	0.1422
Parity, *number*	4.5 ± 2.0	0.0258	4.9 ± 2.3	0.0342	7.3 ± 2.6	0.0064
Delivery Method[3]	-4.2 ± 2.3	0.0753	-2.9 ± 2.7	0.2915	-4.0 ± 3.1	0.2098
Length of storage, *yrs*	5.8 ± 4.9	0.2429	-1.1 ± 5.6	0.8401	28.3 ± 11.4	0.0158
Variability captured (R^2 * 100), *%*	18.95		20.27		44.76	

[1]Birth weight in percentiles is the dependent variable, $P \leq 0.05$. Each biochemical parameter was entered separately in the regression model. The independent predictors were entered based on significant values in the ANOVA and pre-established predictors of birth weight. Only FRAP was a significant positive predictor of birth weight.

[2] For smoking behavior: 0= nonsmoker, 1=smoker

[3] For delivery method: 1= Cesarean section, 2=Induced, 3=Spontaneous vaginal delivery

TABLE 3b. The oxidative stress parameters as independent predictors of gestational age (in wks)[1]

Variable	Gestational age (wks)					
	Model 1		Model 2		Model 3	
	β±SEM	P	β±SEM	P	β±SEM	P
Amniotic fluid						
NO, *μM* (n=192)	0.019 ± 0.007	0.0044				
TBARS, *μM* (n=138)			0.03 ± 0.10	0.7515		
FRAP, *μM* (n=90)					0.0007 ± 0.0008	0.3445
Maternal/Infant Variables						
Parity, *number*	-0.01 ± 0.09	0.9019	-0.10 ± 0.12	0.3977	-0.07 ± 0.09	0.4395
Ethnicity[2]	-0.4 ± 0.12	0.0003	-0.42 ± 0.15	0.0058	-0.33 ± 0.12	0.0061
Delivery method[3]	0.16 ± 0.12	0.1718	0.14 ± 0.15	0.3780	0.28 ± 0.13	0.0345
Length of storage, *yrs*	0.03 ± 0.26	0.9165	0.42 ± 0.32	0.1917	0.49 ± 0.45	0.2851
Variability captured (R^2 * 100), *%*	11.31		9.28		14.48	

[1]Gestational age in wks is the dependent variable, $P \leq 0.05$. Each biochemical parameter was entered separately in the regression model. Only NO was a significant positive predictor of gestational age. Parity, ethnicity, delivery method, and storage length were entered as independent predictors based on variation of the parameter in ANOVA and pre-established predictors of gestational age.

[2] For ethnicity: 1= Caucasian, 2=Asian, 3=Middle-Eastern, Black, and South American

[3] For delivery method: 1= Cesarean section, 2=Induced, 3=Spontaneous vaginal delivery

CHAPTER 6: GENERAL DISCUSSION

Values compared with literature

The determination of NO production by the Griess reaction has been consistently used in biological fluids including plasma and amniotic fluid; however literature values can't be directly compared with our results since some studies measure different metabolites of NO such as nitrite (Egberts *et al.,* 1999) while others have normalized values for creatinine (Tranquilli *et al.*, 2003, Marinoni *et al.*, 2000). Other studies have measured NO synthase (NOS), the enzyme which catalyzes the synthesis of NO from L-arginine, activity in the myometrium and trophoblast cells during pregnancy (Morris *et al.,* 1995a, Myatt *et al.* 1997b). One prospective study (von Mandach *et al.*, 2003) measured amniotic fluid nitrate and nitrite levels in all three trimesters with reported concentrations of 24.87 ± 2.78 μM (n=12) during second trimester, which was slightly lower than our 32.58 ± 1.10 μM. A wide array of markers for lipid peroxidation such as MDA, lipid hydroperoxides, and conjugated dienes have been measured in pregnancy. The use of lipid peroxidation products to assess oxidative stress in second trimester amniotic fluid was first utilized by Burlingame *et al.* (2003); however, the lack of comparative methods prevents comparison with our study. MDA concentration was measured by Bazowska *et al.* (1994) in amniotic fluid and units were expressed as mmol/l as our study did; however samples were obtained during the first stage of parturition. The researchers found the mean MDA concentration in pregnancies complicated by IUGR (0.785 mmol/l) was almost three times higher than normal pregnancy (0.268 mmol/l). The mean concentration of TBARS in our study (3.53 ± 0.11 μM) was comparable to plasma MDA levels (3.0 ± 1.3 nmol/ml) in uncomplicated pregnancy (Pasaoglu *et al.,* 2004). The mean FRAP values (830.4 ± 13.5 μM) were slightly higher than values in 1 month old full term healthy infants (775 ± 196 μM) (Friel *et al.,* 2004). Our study may suggest that the human infant is exposed to oxidative stress as early as second trimester.

Study design limitations

Our study was part of an ongoing biobank collection of amniotic fluid samples from subjects undergoing amniocentesis. The advantage of this approach is it allows us to mimic a large population and increases the ability to detect differences among high-

risk and low-risk groups. In other words, the large sample size increases the power of the study. At the beginning of this investigation, the effect size could not be determined since there were no previous studies that measured NO in amniotic fluid using the same measurement units nor did any measure TBARS and FRAP. After excluding all values with missing maternal or fetal information, a total of 654 samples were available for analysis for NO, 367 for TBARS, and 322 for FRAP. Figure 1 describes the population distributions for the three parameters with our initial sample size. However, as the initial statistical analysis raised the possibility of the influence of sample storage on parameter stability, we proceeded to exclude samples that were stored longer than two years. This reduced our sample sizes to 198, 143, and 94 for NO, TBARS, and FRAP respectively. However, we obtained the same results when performing the analysis by ANOVA on birth weight outcome and gestational age for both the larger n data set and smaller data set which had storage exclusion criteria, which shows that increasing our n did not change the outcome.

With regards to FRAP, we did not have any samples stored less than 1 year that could be measured for total antioxidant capacity due to the fact that the assay was introduced at a later stage in the study, but even with this lower number we still observed a significant relationship between FRAP and birth weight. This did however further limit our sub-groups: GDM and PIH for each parameter, decreasing our statistical power. Hence, the inverse relationship between FRAP and PIH was no longer evident when the PIH group was reduced from nine to two samples after applying storage exclusion criteria. This is illustrated in Figure 2, where the levels of each biomarker were compared between mothers with GDM, PIH, obesity, and smoking behavior vs. those without those conditions. We could no longer test the hypothesis of whether levels of oxidative stress parameters were altered in conditions such as GDM and PIH. Future research is warranted to illustrate the role of oxidative/nitrative stress in maternal disease states.

Development of covariates for birth outcomes

The covariates used to test for associations with birth weight outcome were determined on the basis of previously established associations with birth weight, which included maternal height, prepregnancy weight, ethnicity, parity, infant gender, and

gestational age (Kramer *et al.,* 2001, Kramer, 1987a,b). Differences in concentrations of each biochemical parameter across amniocentesis week were also tested due to previously studied parameters (IGF BP1 and BP3) which were influenced by gestation in amniotic fluid (Tisi *et al.,* 2005). Gestational age was predicted by maternal height and ethnicity previously (Tisi *et al.,* 2004) and hence these variables were included in our analysis. Finally, storage length was included as a covariate when it was found to influence concentrations in our initial analysis.

For the use of birth weight outcomes, we selected the gender-corrected birth-weight-for-gestational-age classification to report results in the paper. This is because the importance of risk assessment based on birth weight distributions by gestational age has been emphasized by authors since the distinction between smallness due to short gestation or to growth retardation may be masked when only birth weight definitions are used (Tentoni *et al.,* 2004). However, we also tested for differences across birth weight categories using the gram classification by ANOVA and regression analysis and obtained similar results to that for gender-corrected birth-weight-for-gestational-age (Table 4,5).

Impact of storage conditions

The determination of the optimal conditions of storage of amniotic fluid samples is necessary when using frozen samples to establish the biochemical composition of the fluid; however the effect of varying storage conditions on analytes in amniotic fluid is not well documented. In this study, we produced a storage distribution of our samples to create a representation of storage length for each parameter (Figure 2). The total number of samples in which storage length was documented consisted of 214 NO samples, 373 TBARS samples, and 322 FRAP samples. In our initial analysis of the entire population, only TBARS differed across storage when entered as a continuous variable; however, both NO and TBARS differed across quartile storage periods. However, once we limited storage length for all three analytes to less than 2 years, we found a significant positive correlation with storage for TBARS concentrations only.

The impact of storage conditions on TBARS levels was studied. Jozwik *et al.* (1999) studied the concentrations of conjugated dienes, lipid hydroperoxides and TBARS in follicular fluid and blood serum in two different determinations, which were conducted

with or without an antioxidant to investigate the peroxidation associated with the methods used. The results revealed that the oxidative stress biomarkers' concentrations in the protected samples (stored at -196°C with addition of antioxidant vs. storage of -20°C without antioxidant) were significantly lower. Hence, the impact of the sample collection and storage in lipid peroxidation products formation was significant. The authors concluded that the measurement of lipid peroxidation through widely used methods may produce inadequate results unless length of storage in included and all future sampling and storage for peroxidation measurements should use the antioxidant/liquid nitrogen method described in the study in order to obtain precise and comparable results.

Zaidman *et al.* (1992) has also studied a variety of metabolites (including alkaline phosphatase, aspartate aminotransferase, glucose, urea, uric acid, creatinine, Na, K, Cl, Ca, P) under different storage conditions. They found that the majority of the analytes were not substantially altered after 7 days of storage at -20°C or at -70°C compared to fresh samples. Only the activity of two enzymes, creatinine kinase and lactate dehydrogensase, were considerably decreased. They concluded that fresh samples are the most adequate for the analysis of amniotic fluid composition; however, suitable conditions may need to be verified for each analyte. Matteucci *et al.* (2001) evaluated the stability of advanced oxidation protein products (AOPP), a useful marker of protein oxidative damage, in plasma. A total of twenty-four samples were processed immediately and after 7, 15, 30, 90, 180, and 438 days of storage both at -20°C and at -80°C. Samples were frozen and thawed only once. The mean concentration of AOPP remained the same for each subsequent determination; however it significantly increased after 438 days of storage, both at -80 °C and considerably at -20°C. They concluded that AOPP remained stable during sample storage for 6 months both at -20°C and -80°C. This may provide us with a method in the future to test aliquots of each biochemical parameter for stability across storage time. Interestingly, they also found AOPP to be positively correlated with TBARS.

Indeed, sample storage and preservation has been one of the problems associated with poor performance of the TBA test leading to many authors heavily criticizing the method (Wasowicz *et al.*, 1993). Many researchers (Lepage et al., 1991, Bonnefont et al., 1989, Lee, 1980) have added antioxidants to the reaction mixture in order to inhibit

metal-catalyzed autoxidation of coexisting PUFAs. Lee (1980) showed that the use of both GSH and EDTA was better than EDTA alone for protecting plasma from lipid peroxidation, but this approach would not be acceptable for our amniotic fluid samples because we are studying the decreases in antioxidant activity in samples exposed to oxidative stress.

Methodological limitations

Numerous methods have been developed to measure lipid peroxidation products as an index of oxidative damage in tissues, cells and body fluids. The measurement of lipid peroxidation by the MDA-TBA method have been used in a variety of diseases; however, this method has been criticized with respect to its analytical specificity, sensitivity, reproducibility, and recovery (Halliwell and Gutteridge, 1990). The assay measures peroxidation products other than MDA which react with TBA, and although the assay is often termed the MDA test by authors, the more accurate description of the method is TBARS. Moreover, a great proportion of MDA can be produced during the determination procedure.

The lack of good markers of lipid peroxidation in vivo has been one of the problems encountered with determining whether increased lipid peroxidation occurs in preeclampsia and other pathological pregnancies. The lack of comparative methods also between studies has prevented researchers from reaching conclusive evidence about the existence of oxidative stress in the preeclamptic state (Llurba *et al.,* 2004).

Bird *et al.* (1983) has described HPLC techniques in which the MDA-TBA adduct is separated from interfering substances by chromatography. Hence, specificity can be greatly improved if combined with HPLC. If levels of TBARS are increased as in the case of our study, then other more sophisticated assays should be performed to verify the results (Esterbauer, 1996). Contrary to lipid hydroperoxides, F2-IPs are chemically stable end products of lipid peroxidation, and the measurement of their levels in plasma lipoproteins may provide a sensitive and specific method for detection of lipid peroxidation damage in vivo (Roberts and Morrow, 2000). Hence, the utilization of isoprostanes as markers of oxidant injury in amniotic fluid can be a future research course.

The advantages of the FRAP assay are that it is easily automated, highly reproducible, linear, and it is not greatly influenced by albumin which can be an

important contributor to antioxidant capacity. The FRAP assay is simple and inexpensive but does not measure the SH-group-containing antioxidants (Cao and Prior, 1993). In fact, it is among various methods developed to assess reductive capacity of body fluids ie. the capacity of body fluids to reduce radicals. However, as reviewed by Dotan *et al.* (2004), the assay results of such potency tests intercorrelate weakly and rarely because there are differences in what these assays measure. Although FRAP has been declared as more direct than other antioxidant assays, its "biological significance" is questioned even if the case is made that some of the oxidative stress in vivo results from Fe-complexes. The FRAP assay has similar shortcomings to the TRAP (Total Radical-trapping Antioxidant Parameter) assay as it measures predominantly uric acid (Abuja and Albertini, 2001). The subtraction of the contribution of uric acid to the "total" antioxidant power can be performed to provide a more sensitive index of antioxidant status in uric acid-rich fluids such as plasma. With regards to amniotic fluid, literature has shown an increase of uric acid concentrations across gestation as more fetal urine is added to the amniotic fluid (Weiss *et al.,* 1974). Mean amniotic fluid uric acid concentrations have been reported as 237.92-1500 μmol/L (Elian KM thesis, 2001). The concentration of uric acid expected in amniotic fluid at 14-16 weeks is 237.92 ± 59.48 μmol/L (Benzie *et al.,* 1974). Since uric acid, albumin, and Fe-complexes naturally exist in amniotic fluid, further research would warrant determining the physiological importance of the contribution of various antioxidants to the total antioxidant power. A different approach for measuring antioxidant status that may be of potential use is to break down total antioxidant capacity into components and separately measure antioxidants such as transferrin and uric acid, which would each contribute a different significance to the overall level of amniotic fluid antioxidant capacity.

In attempt to evaluate the relationship between different assays of oxidative stress, Dotan *et al.* (2004) compared results in studies where oxidative stress has been measured by at least two methods. They found good correlations between the concentrations of breakdown products of lipid peroxidation, such as MDA, F2-IPs, lipid hydroperoxides, conjugated dienes, glutathione, and protein carbonyls but not with other "individual oxidative stress" criteria, including the concentrations of antioxidants (correlations enzyme antioxidants and vitamin E) and DNA fragmentation (correlations between direct

measurements of DNA fragmentation and indices of oxidation-related DNA damage and repair).

Confounding factors

The classification of PIH in our study encompassed all types of hypertensive-related disorders of pregnancy, which may include gestational hypertension, chronic hypertension, and preeclampsia. Since we did not differentiate between these disorders, this could present a limitation especially since endothelial dysfunction is implicated in the pathogenesis of preeclampsia and it represents a more serious clinical disorder. Similarly, in the smoking group, we did not include the amount and duration of smoking in our analysis so that mothers who stopped smoking before pregnancy were amalgamated with mothers who smoked a greater number of cigarettes during pregnancy.

The susceptibility of preeclamptic mothers to lipid peroxidation may result from maternal dyslipidemia, including hypertriglyceridemia, which could occur as early as 20 weeks of gestation (Chappell *et al.,* 2002) causing a rise in free fatty acid concentrations and triglycerides (Gratacos *et al.,* 1996). Moreover, abnormal lipid metabolism may have a role in the pathophysiology of preeclampsia (Hubel and Roberts, 1999) since hyperlipidemia was shown to be enhanced in preeclampsia (Maseki *et al.,* 1981). In future studies, one can further explore the associations between elevated lipid peroxidation markers in preeclamptic women with or without the presence of abnormal lipid profiles. The influence of other pre-existing maternal factors such as diabetes which add to the oxidative stress need to be taken into consideration.

The concentrations of antioxidants may have been influenced by dietary intake; however, this was not assessed in our study. Multivitamin supplements were taken by the majority of the women in our population but this still does not account for dietary sources or tissue stores due to pre-pregnancy supplementation. Since the levels may have been different between subjects taking multivitamins vs. those that were not, the influence of dietary intake and/or supplementation on amniotic fluid levels warrants further investigation, particularly given the contribution of vitamins to the FRAP assay.

The origin of ROS and total antioxidant capacity in the amniotic fluid is still not clear. An understanding of the site of production of these species will help define the role

that oxidative stress plays in various complications of pregnancy such as premature rupture of membranes, IUGR, and preeclampsia. As previous studies have focused on the adverse effects of oxidative stress in pathological states of pregnancy, our study presents novel findings in showing how oxidative stress parameters may change in a largely healthy population during second trimester amniotic fluid. The biochemical analysis of oxidative and nitrative stress parameters in amniotic fluid may potentiate strategies in optimizing fetal growth and development.

FIGURE 1.

Population distribution of amniotic fluid biomarkers NO (n=654), TBARS (n=367), and FRAP (n=322) concentrations. Amniotic fluid NO and TBARS were not normally distributed and hence were $\log_{10}$ transformed.

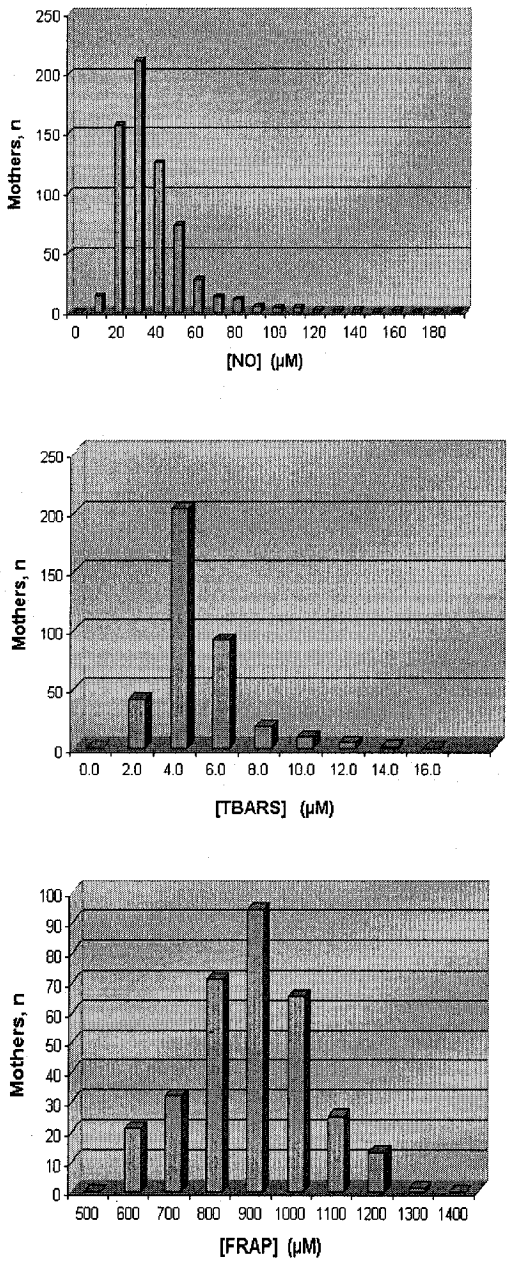
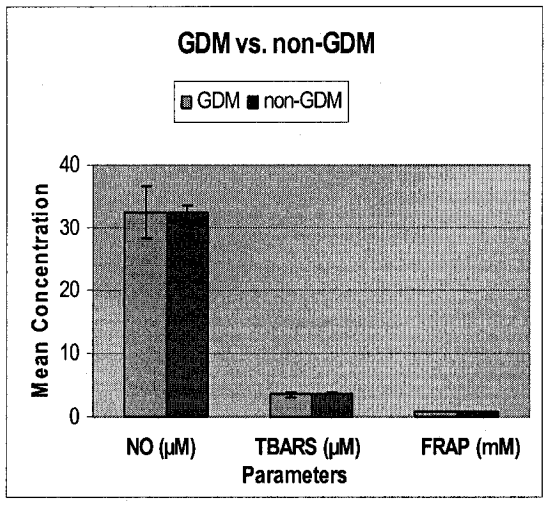
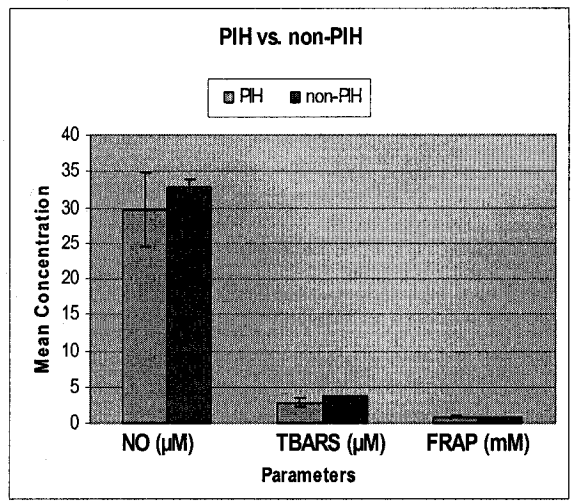
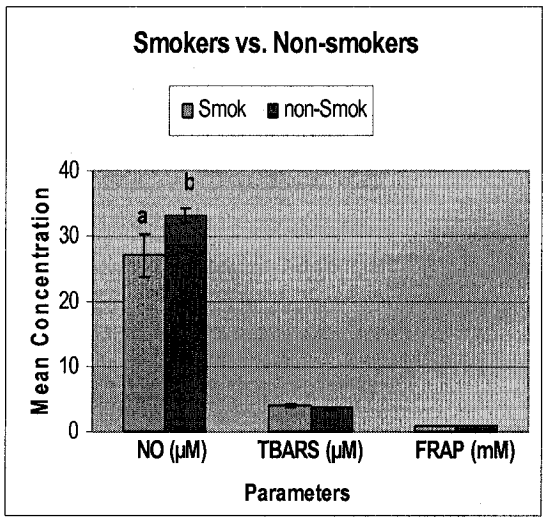


FIGURE 2.

Differences in levels of biomarkers between mothers with GDM, PIH, smokers, and obese and mothers without these conditions for all samples stored below 2 years and corrected for storage







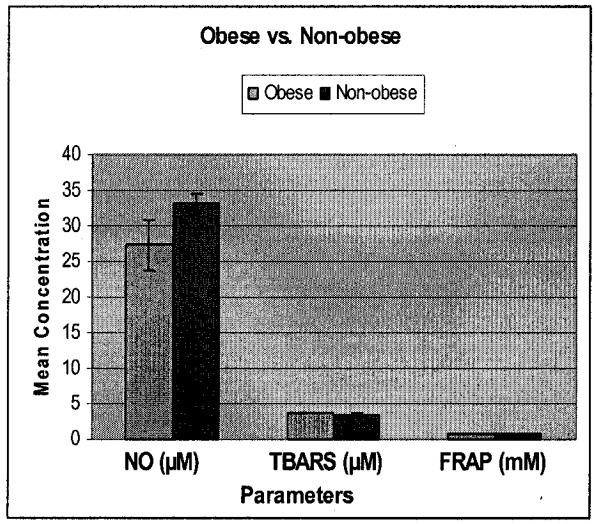
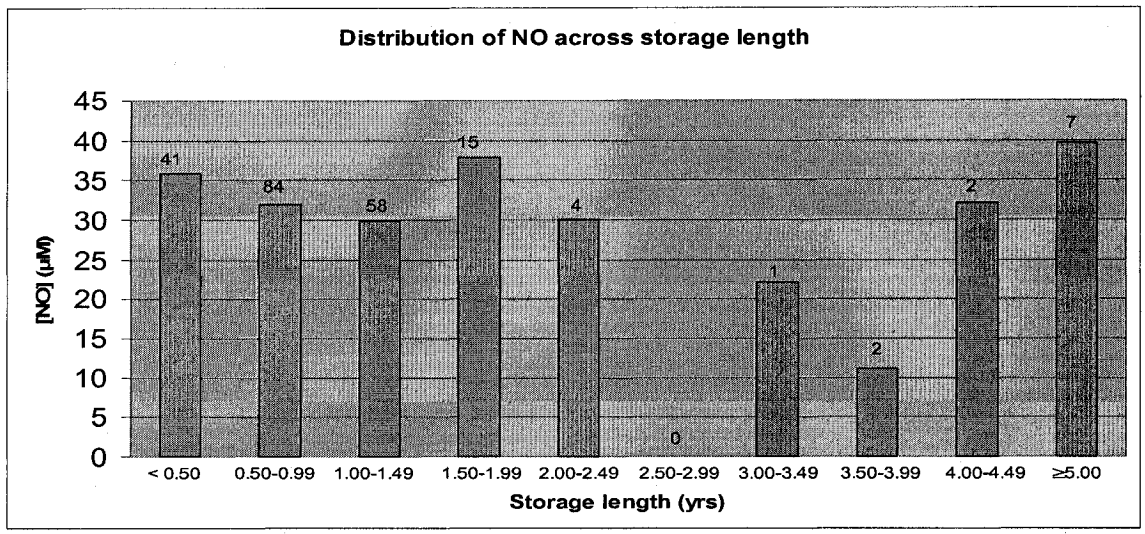
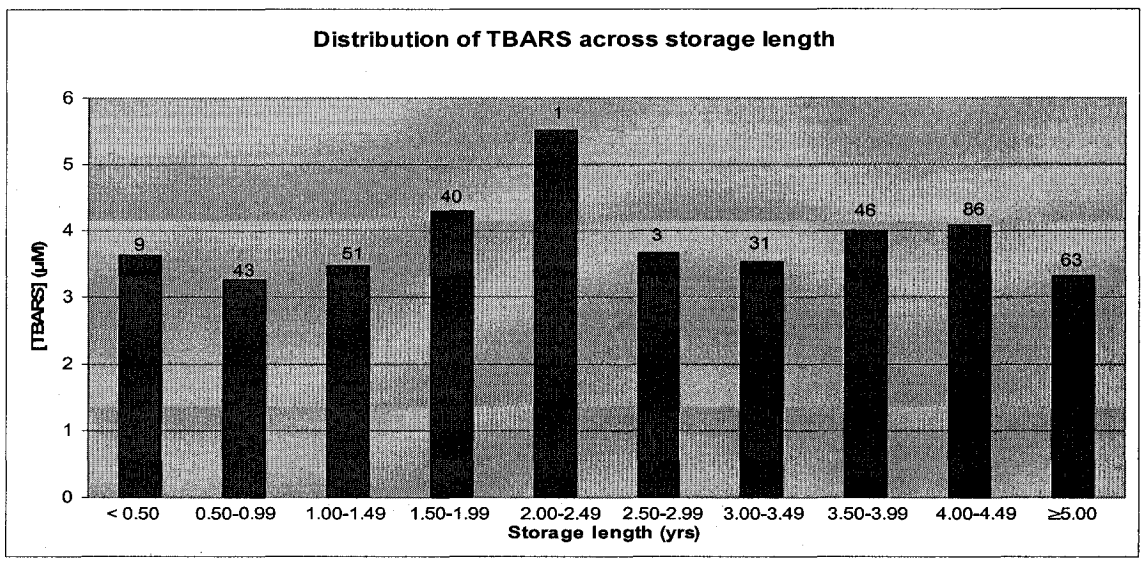


FIGURE 3.
Distribution of amniotic fluid parameters NO (n=214), TBARS (n=373), and FRAP (n=322) across storage length in years.


Distribution of NO across storage length
[NO] (µM)
45
40
35
30
25
20
15
10
5
0
41
84
58
15
4
0
1
2
2
7
< 0.50
0.50-0.99
1.00-1.49
1.50-1.99
2.00-2.49
2.50-2.99
3.00-3.49
3.50-3.99
4.00-4.49
≥5.00
Storage length (yrs)


Distribution of TBARS across storage length
[TBARS] (µM)
6
5
4
3
2
1
0
9
43
51
40
1
3
31
46
86
63
< 0.50
0.50-0.99
1.00-1.49
1.50-1.99
2.00-2.49
2.50-2.99
3.00-3.49
3.50-3.99
4.00-4.49
≥5.00
Storage length (yrs)

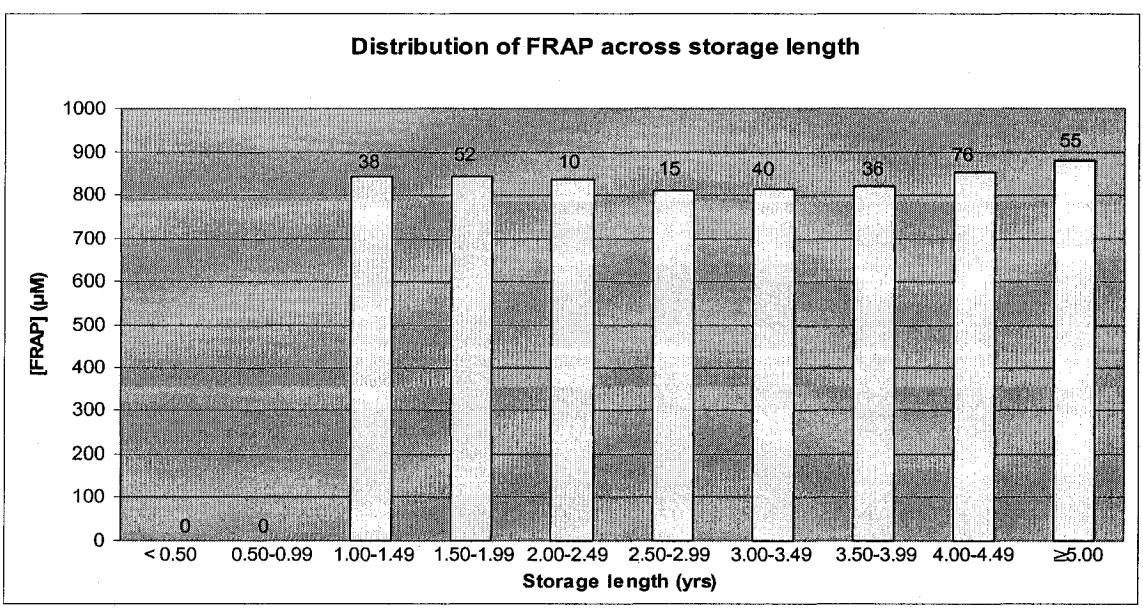

Distribution of FRAP across storage length
[FRAP] (µM)
1000
900
800
700
600
500
400
300
200
100
0
0
0
38
52
10
15
40
36
76
55
< 0.50
0.50-0.99
1.00-1.49
1.50-1.99
2.00-2.49
2.50-2.99
3.00-3.49
3.50-3.99
4.00-4.49
≥5.00
Storage length (yrs)

TABLE 4. Differences in maternal, infant and human amniotic fluid characteristics for the entire population (n=191-198) across birth weights in grams: LBW, normal, and macrosomic[1]

Characteristic	LBW	Normal	Macrosomic	F	P
Infant					
n	4	161	33		
Birth weight, *g*	2004 ± 302[a]	3338 ± 27[b]	4310 ± 49[c]	152.41	<.0001
Gender, *% female*	100.0[a]	49.1[a]	33.3[ab]	3.70	0.0265
Gestational age, *wk*	35.2 ± 1.69[a]	39.3 ± 0.10[b]	40.2 ± 0.20[c]	28.33	<.0001
Maternal					
Height, *m*	1.58 ± 0.006	1.62 ± 0.006	1.64 ± 0.011	1.61	NS
Prepregnancy weight, *kg*	58.1 ± 5.8[a]	62.7 ± 1.1[a]	68.9 ± 2.3[ab]	3.32	0.0382
BMI, kg/m^2	23.2 ± 2.3	23.9 ± 0.4	25.6 ± 0.8	1.61	NS
Ethnicity, *%*	ab	a	b	3.89	0.0221
Caucasian	50.0	58.3	84.4		
Asian	25.0	12.2	6.2		
Other[2]	25.0	29.5	9.4		
Nonsmoking, *%*	100.0	89.7	81.3	1.19	NS
Parity	1.00 ± 0.41	1.16 ± 0.09	1.27 ± 0.16	0.43	NS
Delivery method, *%*				0.25	NS
Cesarean section	25.0	29.8	30.3		
Induced labor	25.0	19.9	30.3		
Spontaneous vaginal delivery	50.0	50.3	39.4		
Amniocentesis week, *wk*	15.8 ± 0.83	15.7 ± 0.10	15.5 ± 0.21	0.40	NS

[1]Values are means ± SEM or %. Means in a row with superscripts without a common letter differ, P ≤0.05.
[2] Other includes Black, Middle-Eastern, and Hispanic.

TABLE 5. The oxidative stress parameters as independent predictors of birth weight (in grams) for samples stored 2 years and less.[1]

Variable	Birth weight (grams)					
	β±SEM	P	β±SEM	P	β±SEM	P
NO, *μM* (n=184)	0.19 ± 2.02	0.9236				
TBARS, *μM* (n=125)			36.1 ± 28.9	0.2147		
FRAP, *μM* (n=74)					0.5 ± 0.3	0.0566
Pregravid weight, *Kg*	8.7 ± 2.5	0.0006	9.5 ± 2.5	0.0003	9.5 ± 2.8	0.0012
Height, *m*	1071.9 ± 452.7	0.0190	930.6 ± 481.2	0.0555	1183.9 ± 505.3	0.0222
Smoking behavior, *0=smoker, 1=non*	81.5 ±102.9	0.4293	-	-	-	-
Parity, *1=1 child*	66.8 ± 31.3	0.0341	76.5 ± 34.1	0.0265	104.9 ± 39.0	0.0092
Gestational age, *wk*	180.6 ± 21.7	<.0001	204.5 ± 20.8	<.0001	198.2 ± 31.9	<.0001
Ethnicity, *1=Caucasian,2=Asian 3=Other*	-	-	-	-	-	-
Delivery Method, *1=Cesarean section, 2=Induced, 3=Spontaneous vaginal delivery*	-	-	-	-	-68.3 ± 49.8	0.1752
Gender, *0=female, 1=male*	210.1 ± 61.3	0.0008	185.4 ± 68.1	0.0074	153.7 ± 77.1	0.0504
Length of storage, *yrs*	123.9 ± 78.6	0.1166	-138.4 ± 84.4	0.1038	366.4 ± 165.6	0.0304
Variability captured (R^2 * 100), *%*	43.44		54.55		58.55	

[1]Birth weight in grams is the dependent variable. Only FRAP showed a significant positive association with birth weight for the entire population. The variables were entered in as independent predictors based on stepwise regressions.

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APPENDIX A

McGill University Ethics Approval

APPENDIX B

St. Mary's Hospital Center Ethics Approval

APPENDIX C

Study Consent Form

APPENDIX D

Maternal Questionnaire

McGill

School of Dietetics and Human Nutrition
Faculty of Agricultural and Environmental Sciences
McGill University
Macdonald Campus

École de diététique et nutrition humaine
Faculté des sciences de l'agriculture et de l'environnement
Université McGill
Campus Macdonald

Tel.: (514) 398-7842
Fax: (514) 398-7739
Website: http://www.agrenv.mcgill.ca/dietetic

21,111 Lakeshore
Ste-Anne-de-Bellevue
Québec, Canada H9X 3V9

Questionnaire

Please answer this brief questionnaire. You are reminded that all the information provided will be kept strictly ***confidential****.*

Name:_____ Telephone number:_____

Date of birth:_____ Height:_____feet_____inches *or* _____meters

Ethnic background:
North American ____ South American ____
European ____ African ____
Middle Eastern ____ Asian ____
Other _____

Number of children I have already given birth to: _____

The following information pertains to this pregnancy only:

Weight prior to pregnancy:_____ pounds *or* _____ kg

I am in my _____th week of pregnancy Due date:_____

Hospital where I will deliver:
Royal Victoria ____
Lakeshore General ____
Jewish General ____
St. Mary's ____
Other _____

Name of Obstetrician/Gynecologist:_____

I am a smoker:
Yes ____
if Yes, while pregnant I smoke _____ cigarettes / day
Yes, but stopped while pregnant ____
No ____

While pregnant, I consume an average of:
0-1 alcoholic drinks / week _____
2-5 alcoholic drinks / week _____
6-10 alcoholic drinks / week _____
11-15 alcoholic drinks / week _____

0-1 cups of coffee/tea / week _____
2-5 cups of coffee/tea / week _____
6-10 cups of coffee/tea / week _____
11-15 cups of coffee/tea / week _____

I am currently taking medication (prescribed by my doctor or over-the-counter): Yes___ No___
If you checked yes, please specify _____

THANK-YOU

APPENDIX E

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